Development of a Novel Dendritic Cell Targeting Vaccine Platform Based on Mouse Hepatitis Coronavirus Multigene RNA Vectors

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1 Summary

With the global eradication of important infectious diseases such as smallpox and poliomyelitis, vaccination campaigns remain one of the most successful public health initiatives. Despite this outstanding progress made in the field of vaccination, there is a crying need for vaccines against other diseases. Millions of deaths per annum worldwide are caused by infections with malaria, tuberculosis and HIV. Efficacious vaccines are lacking against important human viral pathogens such as respiratory syncytial virus (RSV) and hepatitis C virus (HCV). Several vaccine strategies fail simply because of lack of precise delivery of their antigenic genetic cargo into the appropriate immune components capable of driving the desired immune response. DCs are specialized in presenting peptides from processed antigens to $CD4^+$ and $CD8^+$ T cells, self and microbial glycolipids to NKT cells and native antigens to B cells. Therefore efficient targeting of these specialized immune components remains one of several challenges in vaccine development. In addition to targeting the antigenic material to DCs, these cells require the concomitant delivery of adequate maturation signals enabling them to be able to fully activate immune effector cells.

Coronavirus-based vectors are currently considered a promising tool to genetically deliver multiple heterologous genes to specific target cells. They are the largest known positive stranded RNA viruses and can afford a cloning capacity of 6-9 kb. Additionally, their transcription strategy allows for the expression of multiple antigens and immunostimulatory cytokines. These viruses possess potentially high advantage in the field of vaccination in that they infect mainly professional antigen presenting cells (pAPCs) such as dendritic cells (DCs) and macrophages. Accordingly, the major aim of the thesis was the development of a coronavirus-based vaccine system that permits the delivery of antigenic structures to antigen presenting cells, preferably DCs, within secondary lymphoid organs.

In chapter 5.1 of this thesis, the basic methodology for the generation of recombinant coronaviral RNA is described. Based on this methodology, first steps were undertaken to develop a mouse hepatitis virus (MHV)-based system that permits the production of virus-like particles. Chapter 5.2 describes a first series of experiments in which the structural proteins E and M of MHV have been deleted in the vector constructs. A packaging cell line that provides the structural proteins in trans has been developed and rescue of replication-deficient, but propagation-competent MHV particles could be demonstrated. MHV-based VLPs expressing an EGFP-containing antigen cassette were shown to be able to transduce primary DCs in vitro. However, since large scale production of VLPs was not possible using this approach, a workable

protocol for high titer production of MHV-based VLPs was established. Chapter 6.1 describes the construction of a set of murine coronavirus-based vectors which are attenuated by the deletion of all the accessory genes, the gene for the structural protein E and in addition encode an attenuating deletion in the replicase-encoded non-structural protein 1. Stable inducible packaging cell lines capable of providing the virus-deleted structural protein E in trans were generated and high titers of virus-like particles (VLPs) were produced using these E-expressing packaging cell lines. These VLPs were shown to be stable for at least 12 passages and were propagation-deficient in wild-type cells.

Chapter 6.2 describes the generation of a series of VLPs that were designed to mediate the expression of different combinations of transgenes. One set of MHV-based VLPs mediates the expression of a fusion protein consisting of the immunodominant epitope (GP33-KAVYNFATC) of the glycoprotein of lymphocytic choriomeningitis virus (LCMV-GP) and the green fluorescent protein (GFP) in combination with or without the expression of the immune-stimulatory cytokine granulocyte macrophage colony-stimulating factor (GM-CSF). The second pair of MHV-based VLPs mediate the expression of a human melanoma (melan-A) CTL epitope fused to yeast ubiquitin and GFP, again together or without GM-CSF. Efficient targeting of dendritic cells and macrophages with vector-mediated antigen expression could be demonstrated in vitro and in vivo. Single immunization with doses between 10^4 – 10^5 pfu elicited strong and long-lasting protective antiviral and antitumor immunity. In conclusion, this novel vaccine platform mediates the delivery of antigens and immune-stimulatory cytokines to cellular components of the immune system that initiate protective immunity.

2 Zusammenfassung

Die Impfungskampagnen, die zur Ausrottung verschiedener Infektionskrankheiten wie z.B. Pocken oder Poliomyelitis geführt haben, stellen eine der erfolgreichsten Massnahmen im öffentlichen Gesundheitswesen dar. Trotz dieser grossartigen Erfolge auf dem Gebiet der Vakzination, besteht weiterhin dringender Bedarf nach Impfstoffen gegen andere Erkrankungen. Aufgrund des Mangels an wirksamen Vakzinen sterben jährlich Millionen von Menschen an Malaria, Tuberkulose oder HIV. Effiziente Impfstoffe fehlen ebenfalls gegen weitere virale Pathogene, wie das respiratorische Synzitialvirus (RSV) oder das Hepatitis C Virus. Die derzeitigen Impfansätze scheitern bei der Induktion von Immunantworten gegen diese Erreger, da die relevanten Antigene sehr wahrscheinlich nicht an die entsprechenden zellulären Komponenten des Immunsystem geliefert werden. Den wichtigsten Zelltyp in der Immunaktivierungskaskade stellen die dendritischen Zellen (DCs) dar, die darauf spezialisiert sind, prozessierte Peptide den $CD4^+$ und $CD8^+$ T-Zellen zu präsentieren, aber auch mikrobielle Glykolipide für NK-Zellen sichtbar zu machen und native Antigene den B-Zellen zu präsentieren. Daher stellt eine effiziente Belieferung dieser spezialisierten Immunzellen mit Antigenen eine besondere Herausforderung für die Vakzineentwicklung dar. Neben der spezifischen Antigenbeladung, sollten die DCs auch adäquaten Maturationsstimuli ausgesetzt werden, um die vollständige Aktivierungskapazität dieser Zellen zu erhalten.

Coronavirus basierte Vektoren sind vielversprechende Werkzeuge, um spezifische Zielzellen mit Antigenen zu beliefern. Diese Viren sind die Grössten der bekannten, positivsträngigen RNA-Viren und stellen eine Klonierungskapazität von 6-9 kb zur Verfügung. Zudem ermöglicht ihre Transkriptionsstrategie die Expression verschiedener Antigene und immunstimulatorischer Zytokine. Die Viren zeigen den weiteren Vorteil, dass sie bevorzugt professionelle Antigen präsentierende Zellen, DCs und Makrophagen, infizieren. Daher war das Hauptziel dieser Arbeit die Entwicklung eines Coronavirus basierten Systems, das den Transport von Antigenstrukturen hin zu Antigen präsentierenden Zellen, bevorzugterweise DCs, in sekundären lymphatischen Organgen gewährleistet.

Im Kapital 5.1 dieser Arbeit wird die grundlegende Methodologie für die Generierung von rekombinanter Coronavirus RNA beschrieben. Basierend auf dieser Methodik wurden erste Schritte unternommen, um ein auf dem Maushepatitisvirus (MHV) basierendes System zu etablieren, das die Produktion von Virus ähnlichen Partikels (VLPs) ermöglicht. In Kapitel 5.2 wird eine erste Serie von Experimenten beschrieben, die die Deletion der Strukturproteine E und M des MHV in den Vektorkonstrukten zum Ziel hatten. Eine Verpackungszellinie wurde

etabliert, die eine Komplementierung der fehlenden Strukturproteine in trans ermöglichte. Mit Hilfe dieser Zellinie wurde die Produktion von replikationsdefizienten, aber propagierungskompetenten VLPs möglich. Es konnte gezeigt werden, dass MHV-basierte VLPs, die eine Antigenkassette mit GFP exprimieren, primäre DCs in vitro transduzieren können. Da aber eine Produktion von grösseren Mengen von VLPs in diesem Ansatz nicht möglich war, wurde ein Protokoll entwickelt, dass die Generierung von hohen Titern der MHV-basierten VLPs ermöglicht. In Kapital 6.1 wird die Konstruktion eines Satzes von MHV-basierten Vektoren beschrieben, bei denen eine Attenuierung durch die Deletion aller akzessorischen Gene und des Gens für das Strukturprotein E und die funktionelle Ablation des Nichtstrukturproteins nsp1 erreicht wurde. Induzierbare Verpackungszellinien, die das virale Protein E in trans bereitstellen, wurden generiert und grosse Mengen an VLPs konnten mit Hilfe dieser Verpackungszellinien hergestellt werden. Diese VLPs waren stabil über mindestens 12 Passagen und waren propagierungsdefizient in Wildtypzellen.

Im Kapitel 6.2 wird das Design einer Serie von VLPs beschrieben, die verschiedene Kombinationen von Transgenen tragen. Das erste Paar dieser VLPs vermittelt die Expression eines Fusionsproteins bestehend aus dem immundominanten Epitop (gp33-KAVYNFATC) des Glykoproteins des Lymphozytären Choriomeningitis Virus (LCMV-GP) und dem grün fluoreszierenden Proteins (GFP), allein oder gemeinsam mit dem immunstimulatorischen Zytokine Granulozyten-Makrophagen-Kolonie-stimulierender Faktor (GM-CSF). Der zweite Satz von Vektoren kodiert für ein humans CTL Epitop des Melan-A Proteins, das mit Hefeubiquitin und GFP fusioniert ist. Auch diese Antigenkassette wurde entweder allein oder zusammen mit GM-CSF verwendet. Effiziente Tranduktion von DCs und Makrophagen mit Vektor vermittelter Antigenexpression in vitro und in vivo konnte demonstriert werden. Einmalige Immunisierung mit Mengen von $10^4 – 10^5$ pfu führte zu starker und langanhaltender, protektiver, antiviraler und antitumoraler Immunität. Zusammenfassend kann festgehalten werden, dass diese neu entwickelte Vakzineplattform eine Lieferung von Antigenen und immunstimulatorischen Zytokinen zum Wirkort einer erfolgreichen und protektiven Immunanwort ermöglicht.

3 Introduction

3.1 The immune system in general

The provision of immunity towards invading pathogens, infectious diseases and cancer often entails a highly orchestrated crosstalk between both arms of the immune system namely the innate and the adaptive immune systems. The innate immune system reacts rapidly within minutes and distinguishes pathogens from self-components through the use of a wide variety of receptors such as Toll-like receptors (TLRs), pattern recognition receptors (PRRs) properly adapted to detect evolutionary conserved signatures from pathogens coined pathogen associated molecular patterns (PAMPs) (Barton and Medzhitov, 2002; Gordon, 2002; Iwasaki and Medzhitov, 2004). The innate immune system baptized as the body's first line of defence is armed with physical, anatomical, physiological, phagocytic or endocytic and inflammatory barriers that provide the organism with such effective system to prevent entry and or colonization by pathogenic microorganisms. However, these nonspecific defence mechanisms devoid of any immunologic memory, cannot always eliminate infectious agents and there are several pathogens that cannot be recognized for various reasons. Innate immune responses, among their many effects lead to a rapid burst of inflammatory cytokines wiring or coding in, the activation status for antigen presenting cells such as macrophages and dendritic cells (DCs) (Pashine et al., 2005) and thereby assist in shaping the upcoming adaptive immune response.

The components of the adaptive immune system have evolved to provide a more versatile means of defence that in addition provides a heightened protection from a subsequent re-infection with the same pathogen. Endowed with the properties of antigenic specificity, diversity, immunologic memory and the ability to segregate self from nonself (Zinkernagel and Doherty, 1974), the adaptive immune system typically lags for about five to six days after initial exposure to a particular antigen.

The adaptive immune system is generally comprised of two arms, the cytotoxic immune response and the humoral immune response. Cytotoxic T lymphocytes (CTL) eliminate infected cells or tumour cells through direct action on the target cells by secretion of perforin and granzyme whereas B cells are the main players in humoral immune response and they mediate the production of antibodies against pathogen-derived molecules. Both processes are dependent on $CD4^+$ T cell help. Furthermore, the T-cell receptor (TCR) on the surface of the CTL or T helper (T_H-) cells forms a complex with the MHC I/peptide epitope complex or the MHC II/peptide-epitope complex, respectively and both reactions are aided by the CD8 or CD4 co-receptor ligation respectively. Since the goal of vaccination is to induce immune responses that

can control an infection or cancer, the vaccine should be able to selectively stimulate antigen-specific CTLs or B cells and T_H cells. Better still if the vaccine can stimulate a combination of all.

The proper functioning of the adaptive immune response involves two major groups of cells, lymphocytes and antigen presenting cells. The induction of both humoral and cell-mediated adaptive immune response depends on the engagement of naive $CD4^+$ T cells and their subsequent activation leading to the production of $CD4^+$ T_H cells.

3.1.1 Antigen presenting cells (APC)

A major difficulty for an effective vaccination strategy is the delivery of the intended antigenic cargo to specific cellular components of the immune system endowed with the potential of steering an efficacious immune response. Furthermore, the effective recognition of antigens by lymphocytes necessitates adequate antigen processing and presentation within specialized complexes which enables specific receptors on lymphocytes to recognise and ligate the so called antigenic determinants or epitopes representing the immunologically active areas of a complex antigen.

Professional antigen presenting cells (mainly B cells, macrophages and dendritic cells) (Lassila et al., 1988; Steinman, 1991; Steinman and Cohn, 1973) are cells of hematopoietic origin capable of sampling their environment for antigens either by phagocytosis, endocytosis, pinocytosis for soluble antigens or by receptor mediated internalization and thereafter migrate to secondary lymphoid organs. These specialized cells are able to express MHC class II and MHC class I molecules on their surface and are equally able to deliver the necessary co-stimulatory signal for T_H cell or T cytotoxic (T_C) cell activation. The internalized antigen is degraded into defined antigenic peptides which are bound to MHC molecules and transported to the cell surface for subsequent presentation. In order for macrophages to function as professional antigen presenting cells, they must be activated by phagocytosis of pathogens before they express the necessary MHC class II molecule or the co-stimulatory B7 membrane molecule. In contrast, B cells express MHC class II molecules constitutively but need to be activated to express the B7 co-stimulatory molecule. In fact, the first reports of B cells as stimulatory APCs involved B cell tumour lines and normal B cells stimulated with anti-Ig (Chesnut and Grey, 1981). The main feature of B cells as APCs is their ability to use their clonally distributed antigen receptors to pick up antigen into their processing machinery. Antigen processing and presentation mediated by membrane Ig was first demonstrated using rabbit Ig-primed T cells, resulting in T cell

proliferation (Chesnut and Grey, 1981). This receptor-mediated specific antigen uptake and presentation has proven to be more efficient than nonspecific antigen presentation (Lanzavecchia, 1990). The remarkable efficiency in antigen presentation by antigen-specific B cells is highlighted, at least in part, by the degree of specificity of the antibody response.

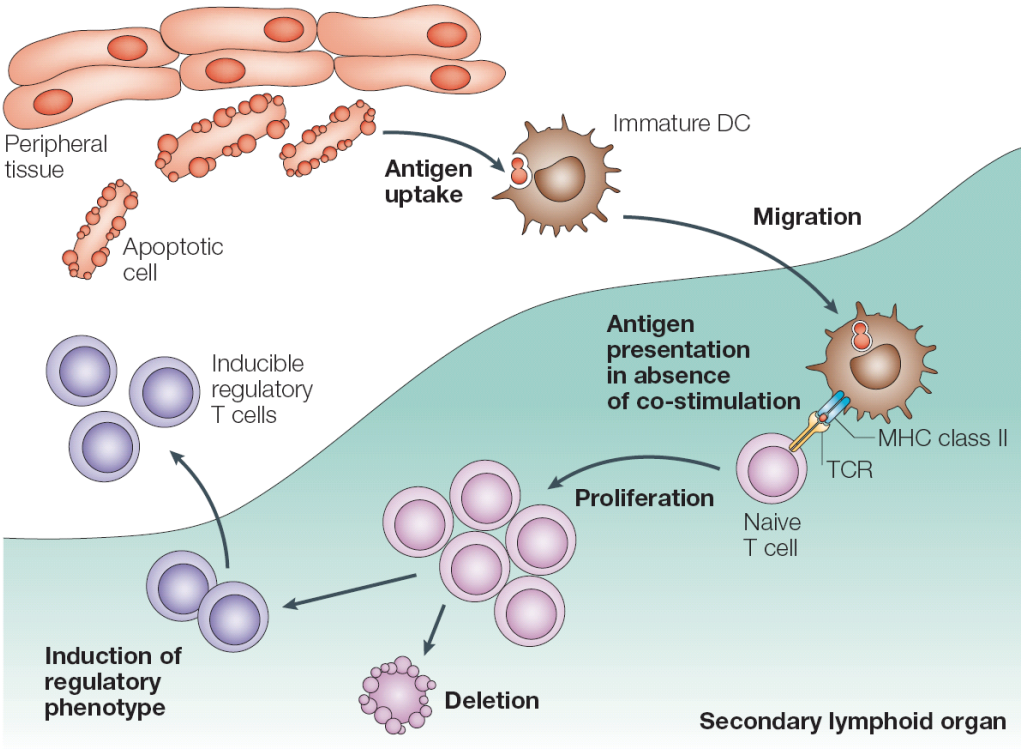

Figure 1: Immature dendritic cells (DCs) induce tolerance. Tissue DCs constantly sample their environment, capture antigens and migrate to draining lymph nodes. In the absence of inflammation, DCs remain in an immature state and antigens are presented to T cells in the lymph node without co-stimulation, leading to either the deletion of the cells or the generation of inducible regulatory T cells (Banchereau and Palucka, 2005).

Finally, dendritic cells, the most potent antigen-presenting cells with a constitutively high density of surface MHC class II and co-stimulatory-adhesive molecules (Delon et al., 1998) display an extraordinary capacity to stimulate naive T cells and initiate primary immune responses. The un-matched capacity of DCs to sample their surrounding, transport apoptotic cellular components as well as antigenic entities to secondary lymphoid organs where they make specific immunologic contacts with T cells make them an ideal target for a successful vaccine approach (Banchereau and Steinman, 1998). In fact, the functional diversity of these ‘’master planner’’ cells, could be traced to the different DC subsets and partly on the functional plasticity at the immature stage (Liu, 2001). Immature DCs (figure 1) characterised by their high rate of antigen capture and low MHC class II expression can be regarded as the security guards of the

body's immune system. They are available at all potential entry portals, highly alert to capture any intruder and ready to relay specific information concerning the intruder to appropriate quarters for subsequent action.

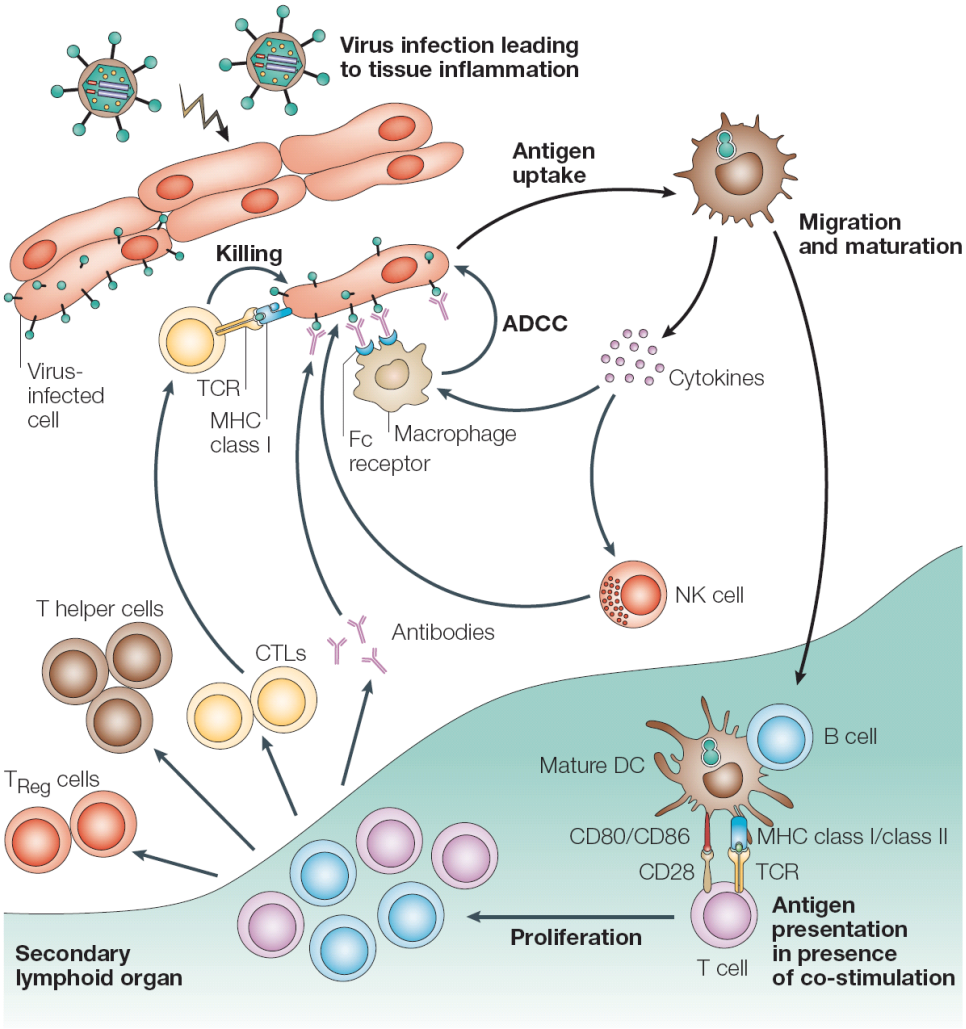

Figure 2: Mature DCs induce immunity. Tissue inflammation induces maturation of DCs and the migration of mature DCs to draining lymph nodes. The mature DCs express peptide-MHC complexes at the cell surface as well as appropriate co-stimulatory molecules. This allows the priming of $CD4^+$ T helper cells and $CD8^+$ cytotoxic T lymphocytes (CTLs), the activation of B cells and the initiation of an adaptive immune response. To control the immune response, $CD4^+$ $CD25^+$ regulatory T (T_{REG}) cell populations are also expanded. ADCC, antibody-dependent cell-mediated cytotoxicity; NK, natural killer; TCR, T-cell receptor (Banchereau and Palucka, 2005).

Furthermore, since DCs can present self antigens and because they persistently provide tolerogenic signals to T cells, they have been nick named the ''police'' of the immune system (Mays and Chen, 2007; Steinman et al., 2000; Villadangos and Schnorrer, 2007). This is compounded by the fact that, immature DCs continuously pick up apoptotic cell debris as well as other self antigens and present them to T cells. Interesting enough, under normal physiological

circumstances after capturing self antigens, these immature DCs do not assume immunogenic form of maturation. In fact, the versatility of DCs in shaping or reshaping the outcome of an immune reaction has been elegantly reported indicating the capacity of DCs to initiate peripheral tolerance by specific interactions with $CD4^+$ and $CD8^+$ T cells (Desai et al., 2007; Luckashenak et al., 2008; Probst et al., 2005; Seewaldt et al., 2002) or by inducing the differentiation of IL-10 producing $CD4^+$ cells with regulatory or suppressor properties (Dhodapkar et al., 2001; Jonuleit et al., 2000).

Presentation of antigen by mature dendritic cells depends on the strength and quality of the activation signals received during antigen sampling. Since immature dendritic cells have various pattern recognition receptors enabling them to recognize and distinguish pathogenic antigenic cargo, they properly decode the signals emanating from various activating reactions. Thus, the presence of pattern recognition ligands (PRL) within endocytosed or phagocytosed cargo provides decisive stimuli to antigen capturing immature dendritic cells picking up such PRL loaded material to rapidly undergo maturation into immunogenic forms (Blander and Medzhitov, 2006). Therefore, immature DCs mainly function as antigen capturing cells whereas mature DCs are specialized in antigen presentation. In vivo, the maturation of DCs is closely linked to their migration from the periphery where they scan for antigens to the draining lymphoid organs where they make contacts with T cells (figure 2).

Maturing DCs are associated with down regulation of endocytic or phagocytic receptors and the expression of co-stimulatory molecules like CD40, CD58, CD80 and CD86, changes in morphology and lysosomal compartments with down regulation of CD68, expression of LAMP3 (lysosomal-associated membrane protein 3) and changes in MHC class II compartments. Additionally, they produce and secrete pro-inflammatory cytokines such as interleukin-1 (IL), IL-6, IL12, IL-18 and IL-23 but also turn on ELC/MIP-3 beta and SLC/6Ckine following the up-regulation of the chemokine receptor CCR7 which promotes migration to draining lymph nodes where mature DCs loaded with MHC class I or II peptides make specific immunogenic contacts to T cells (Caux et al., 2000; Geijtenbeek et al., 2000).

In fact, the combination of IL-1β and TNF with type I and II IFNs seems to yield more potent DCs in terms of secretion of IL-12 and induction of tumour specific CTLs *in vitro* (Mailliard et al., 2004). Additionally the excellent immune-stimulatory capacity and the ability to decode specific molecular pathogenic signals make DCs an efficient means to initiate better antibacterial, antiviral or anti tumour immune responses (Banchereau et al., 2001; Steinman and Pope, 2002). DCs can be marshalled for the treatment and prevention of cancer because tumours are replete with potential antigens and they can become immunogenic if presented by DCs

(Banchereau and Palucka, 2005; Figdor et al., 2004), meaning the immune attack on cancer can be broad enough to encompass multiple targets including mutant proteins expressed by the cancer and not just a single target which may favour immune escape. DCs have a crucial role in determining the type of response that is induced. It has been shown that either polarized DCs or distinct DC subsets might provide T cells with different signals that determine the type of immune response (Shortman and Liu, 2002). Splenic $CD8\alpha^+$ DCs in mice prime naïve $CD4^+$ T cells to produce T_H1 cytokines in an IL-12 involving process meanwhile, splenic $CD8\alpha^-$ DCs prime naïve $CD4^+$ T cells to produce T_H2 cytokines (Maldonado-Lopez et al., 1999; Pulendran et al., 1999). Furthermore, the polarization into different T-cell subsets also depends on the information received by a DC (Pulendran et al., 2001).

Additionally, DCs can activate and expand the different arms of cell-mediated resistance such as natural killer, NKT, γδT and αβT cells each of which recognizes different alterations in cancer cells. The immunogenicity of antigens delivered by DCs has been shown in patients with cancer (Davis et al., 2003; Nestle, 2000) or chronic HIV infection (Lu et al., 2004), thereby providing proof of principle that using DCs as vaccines can work.

Preclinical experiments in mice have demonstrated that anti-tumour activity can be induced using DCs exogenously pulsed with tumour peptides (Ossevoort et al., 1995; Zitvogel et al., 1996), incubated with crude tumour cell lysates (Ashley et al., 1997), transfected with total RNA derived from tumour cells (Boczkowski et al., 1996) or even infected with recombinant viruses (Song et al., 1997; Specht et al., 1997). The translation of these preclinical experimental experiences into clinical trials have demonstrated that the use of DCs is safe and that some significant clinical efficacy can be obtained using this method. However, some important limitations impeach these approaches such as the possibility of tumours to escape from specific immune responses if too few antigens are presented (Nestle, 2000), the suboptimal delivery of cellular antigens to DCs or inappropriate co-stimulation (Brossart et al., 2000).

Actually, the efficacy of DC vaccines can be greatly improved if appropriate signals to induce DC maturation and effective activation of T cells are provided. It may thus be efficacious to target a combination of antigens and immune-activators to DCs (Figdor et al., 2004). These activators should be such that increase the expression of antigen-binding receptors, antigen processing, major histocompatibility complex molecule synthesis and chemokine receptor expression in DCs. Important inducers of maturation are most of the pathogen-derived products (such as LPS, viral dsRNA, and CpG DNA), T cell derived stimuli (CD40), secreted pro-inflammatory stimuli (IL-1 and 2) and tumour necrosis factor (TNF)-α. Interferon-γ (IFN-γ)

could be used to generate mature DCs for use in vaccines where a long lasting memory $CD8^+$ response is needed (Dubois et al., 2002). Furthermore, some workers fused the antigen to heat shock proteins that enters DCs via CD19, activating the DCs and targeting the antigen to the MHC processing pathway (Srivastava, 2002; Srivastava, 2000). Additionally, the maturation of DCs via Toll-like receptors has been demonstrated to enhance cytomegalovirus and HIV specific T cell responses *in vitro* (Lore et al., 2003).

It would be interesting to identify stimuli that produce a desired DC-maturation programme that could lead to the induction of tumour-specific CTLs while inhibiting the induction of T_{Reg} cells in various human DC subsets. For example, Toll-like receptor in combination with a T-cell like signal delivered through CD40 might enhance DC function (Reis e Sousa, 2001). Indeed, TLR-mediated signals are involved in the control of $CD4^+$ T cell activation (Pasare and Medzhitov, 2004). Interestingly, the differential expression of TLRs on distinct DC subsets (TLR-9 pDCs, TLR-2 and TLR-4 myeloid DCs) (Kadowaki et al., 2001), might confer distinct maturation signals yielding distinct types of immune response.

Additionally, distinct maturation and or activation signals (such as prostaglandin E_2) (Luft et al., 2002; Scandella et al., 2002) might induce the preferential expression of CC-chemokine receptor 7 (CCR7) by DCs, thereby increasing the capacity of these DCs to respond to the appropriate ligands CC-chemokine ligand 19 (CCL-19) and CCL21, which are expressed in lymphatic vessels and secondary lymphoid organs (Sallusto et al., 1998b).

3.1.2 T Lymphocytes

The recirculation of naïve T lymphocytes in search of antigen displayed on antigen-presenting cells, through the blood stream and the lymphoid organs is orchestrated by adhesive effects between lymphocytes and cells of the endothelium (Picker and Butcher, 1992). In principle, the expression of different sets of chemokine receptors, integrins and selectins provide lymphocytes with a precise navigation system for exit at specific sites and retiring at various locations in the body (Butcher et al., 1999; Campbell and Butcher, 2000; Sallusto et al., 1998a). The initiation of any T cell immune response emanates within the secondary lymphoid organs with the establishment of contact between the T cell and antigen-presenting cell representing signal 1 followed by binding of an adequate co-stimulation representing signal 2 (Salomon and Bluestone, 2001) . This contact takes place in a central space termed the immunological synapse surrounded by adhesion molecules in which the T cell receptor (TCR) and the co-stimulatory molecules congregate (Dustin and Cooper, 2000). Engagement of the TCR by MHC-peptide

complexes at the immunological synapse initiates some kind of conformational change or aggregation of the TCR-CD3-ζ (Lanzavecchia et al., 1999) complex releasing a signaling cascade of which the duration and strength dictates entry of the naïve T cell into the cell cycle (Lanzavecchia and Sallusto, 2001). In principle, the efficiency of the immunological synapse to function as a signal transduction platform depends on the developmental stage of the T cell and the activation status of the particular APC. After this primary interaction of the TCR with its specific peptide-mounted MHC, the expression of CD28 and CD40L is turned on. The non-antigen specific co-stimulatory signal is provided by engagement of one or more T cell surface receptors CD40 or CD28 with their respective ligands CD40L, B7.1 (CD80) and B7.2 (CD86) on APC. Though the particular cytokine microenvironment may influence naïve T cell differentiation, it has been demonstrated that the strength of the TCR signal may determine the co-stimulatory requirements for Th1 and Th2 $CD4^+$ T cell polarization (Tao et al., 1997). In fact, T cells that receive a prolonged TCR stimulation in the presence of IL-4 or IL-12 may be committed and terminally differentiate into Th1 cells producing IFN-γ or differentiate to Th2 cells producing IL-4, IL-15 and IL-13 (Abbas et al., 1996; O'Garra, 1998; Seder and Paul, 1994). After their activation, differentiated T cells extravasate to the inflamed (Campbell et al., 1998) peripheral nonlymphoid tissue as effectors guided by the expression of adhesive ligand molecules such as L-selectins, LFA-1, VLA-4 and Mac-1 which interacts with activated adhesive molecules of the endothelium such as P- and E-selectins VCAM-1 and ICAM-1.

Furthermore, this activation phase may lead to up-to 1000-fold expansion of the primary effector cells within a few days (Goldrath and Bevan, 1999). However, after the invading pathogen such as LCMV has been checked, more than 90% of the expanded primary effector T cells are eliminated in the second phase by apoptosis (Lin et al., 2000) via FasL-Fas or TNF-a-TNF-R mediated interaction (Zheng et al., 1995). This massive down regulation is necessary to maintain homeostasis and prevent autoimmunity (Lenardo et al., 1999). The final phase is composed of long-lasting ‘’memory’’ T cells that maintain their antigen specificity and can persist later on in reduced numbers for the life time of the host (Sallusto et al., 2000; Sprent and Surh, 2001; Zinkernagel, 2000).

Interestingly, memory T cell burst in an immune response as compared to the primary response is characterised by its ability to rapidly generate larger number of antigen specific T-helper cells and CTLs. Studies in mice suggest that $CD8^+$ memory CTLs are derived from the same initial pool of expanding effector T cells (Kaech and Ahmed, 2001; Mercado et al., 2000; van Stipdonk et al., 2001) and these CTLs express effector molecules like granzyme B (Jacob and Baltimore, 1999) , perforin (Opferman et al., 1999) and cytokines (Saparov et al., 1999).

There is growing evidence suggesting that naive, effector and at least two types of memory T cell population namely central-memory and effector-memory T cells exist in mice and human (Campbell et al., 2001; Potsch et al., 1999; Reinhardt et al., 2001; Sallusto et al., 1999). Some workers have demonstrated that during the activation phase, cycling $CD8^+$ T cells are $CD27^+$ in contrast to terminally differentiated perforin ripe CTLs which are $CD27^-$ (Hamann et al., 1997; Roos et al., 2000), suggesting the loss of CD27 could be a marker for effector and effector memory CTL (Campbell et al., 2001; Hamann et al., 1997). Based on the homing characteristics and their effector function, two types of memory T cells have been described within the $CD4^+$ and $CD8^+$ T cell population. T central-memory (T_{CM}) cells express CD62L and CCR7 whereas T effector memory (T_{EM}) cells do not express CCR7 and CD62L (Sallusto et al., 1999). Indeed, in humans and mice, $CD4^+$ T_{CM} and $CD8^+$ T_{CM} locate within the lymphoid organs (Masopust et al., 2001; Reinhardt et al., 2001; Sallusto et al., 1999). Upon restimulation, $CD4^+$ T_{CM} divide rapidly and secrete IL-2 and IL-10 (Reinhardt et al., 2001; Sallusto et al., 1999) whereas $CD8^+$ T_{CM} retains ability to secrete IL-2 but needs re-priming to regain perforin killing function and IFN-γ production (Masopust et al., 2001).

There is sufficient evidence that $CD8^+$ T cells autonomously divide and acquire cytotoxic function in the absence of further antigen stimulation when once they have been committed, thus elaborating an imprinted clonal expansion and differentiation instruction in naïve $CD8^+$ T cells during a brief encounter with the antigen-presenting cell (Kaech and Ahmed, 2001; van Stipdonk et al., 2001).

In summary, memory T cells are very important components of the immune system and form the basis for the success of many vaccines because the T_{EM} cells provide immediate protection against re-infection or reactivation of disease at sites of infection whereas T_{CM} cells residing primarily in the lymphoid tissue can rapidly expand and differentiate to resupply the effector T cells at the periphery.

3.1.3 B cells and neutralizing antibodies

The humoral immune response begins when antigen cross-links membrane-bound antibody molecules on B cells. Some of these antigens become internalised by receptor mediated endocytosis, shuffled for procession through the endocytic pathway and combined to MHC class II molecules which are subsequently transported and presented on the B cell membrane. Mature naive B cells that have picked up antigen and are activated, must travel towards the interface between the primary follicle and the T-cell zones in order to present their antigenic cargo to

antigen specific T helper cells (Monson, 2008). The initial priming of antigen specific $CD4^+$ T cells requires signals mediated by interactions with ligated MHC class II-peptide complexes and members of the B7 family of co-stimulatory molecules (Constant, 1999). This binding activates the $CD4^+$ T helper cells to secrete cytokines such as IL-2 and express surface molecules such as CD40L (Banchereau et al., 1994; Oxenius et al., 1996; Parker, 1993) that are important stimuli of B cell proliferation and differentiation into memory B cells and effector or antibody secreting plasma cells. Additionally, anti-lipid antibody production by B cells has been shown to involve help from a CD1d-instructed lipid-specific invariant natural killer T cell (Florence et al., 2008; Leadbetter et al., 2008; Speak et al., 2008). Long-lived antibody-secreting plasma cells may also contribute to immunological memory (Manz et al., 1997). Furthermore, the quality, strength and rapidity of memory responses are often different from the primary immune response to antigen. Memory B cells express membrane immunoglobulins of the IgA, IgE and IgG classes on their surface allowing quick recognition followed by reaction to free antigen (Julius et al., 1972).

Existing evidence implicate basophils and antigen-specific B cells to be the only leukocyte populations that can bind substantial amounts of intact antigen on their surface after immunization (Mack et al., 2005). Basophils trap antigen-specific IgE antibodies present in the plasma after immunization by expression of the high affinity IgE receptor (Mack et al., 2005). These cells have been shown to induce a T_H2 phenotype in $CD4^+$ T cells *in vitro* (Hida et al., 2005; Oh et al., 2007) and *in vivo* (Sokol et al., 2008). Recognition of the appropriate antigen specific to the bound IgE antibodies months after immunization demonstrates the long term persistence of an antigen-specific IgE response, suggesting that basophils may be involved in the development of memory immune responses. More recent reports actually implicate basophils to profoundly enhance B cell proliferation and immunoglobulin production in a $CD4^+$ T-cell-dependent manner (Denzel et al., 2008), summarising that basophils could be important contributors to humoral memory immune responses.

The effector function of plasma B cell secreted antibodies is mediated by binding to antigens and neutralizing them and or facilitating their elimination by various means including phagocytosis, complement activation and opsonization.

In fact, the importance of B cells in the generation of neutralizing and non-neutralizing antibodies cannot be under-estimated in assuring immunity against certain viral pathogens. The ease of generating neutralizing antibodies varies extremely and convincing arguments that repetitive viral surface antigens induce the most potent antibodies to virions have been brought to book (Bachmann and Zinkernagel, 1996, 1997). For example the densely packed, highly organized glycoprotein of vesicular stomatitis virus (VSV-G) is capable of inducing a strong

neutralizing T help independent antibody response whereas its soluble form as an immunogen fails to compete (Bachmann and Zinkernagel, 1996). Therefore, a native form of VSV either attenuated or killed should make a good vaccine from an antibody point of view. In principle antibodies can act against free virus as well as virus infected cells.

The term neutralization defined as loss of infectivity results when antibodies bind to a virus particle and prevent its ability to infect a cell. The binding of antibodies to virus infected cells can lead to several antibody mediated antiviral immune properties. Most fundamental of all is that binding of antibodies to virus infected cells can effectively inhibit release of progeny virions (Gerhard, 2001) and thereby preventing cell-to-cell transmission (Burioni et al., 1994; Pantaleo et al., 1995). The binding of antibodies on viral proteins or molecules expressed on the membranes of infected cells, could through signal transduction initiate mechanism that inhibit virus replication inside the cells as demonstrated in virus infected neurons (Fujinami and Oldstone, 1979; Levine et al., 1991).

Furthermore, the antibody Fc-mediated effector activity can lead to antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). Additionally, antibody-Fc mediated effector functions can affect the activity of free virus particle in the organism. The Fc and complement receptor can bind antibody or complement coated virions leading to phagocytosis followed by inactivation of the virion in an intracellular compartment of the phagocyte. This is believed to be an important *in vivo* mechanism against Foot and mouth disease virus (FMDV) (McCullough et al., 1988).

It should be recalled that vaccine –induced antibodies that interfere with viral entry and spread are the protective correlates of most existing prophylactic vaccines today. However, the situation remains elusive with highly variable viruses such as HIV-1 where the ability to induce neutralizing antibodies by vaccination has proven to be futile.

3.2 History of Vaccination

The act of variolation or the deliberate induction of immunity originally used by the Chinese and Turks was greatly improved by the British physician Edward Jenner in 1798. Surprised by the fact that the milk maids who had contracted cowpox were eventually immune to smallpox, Jenner reasoned that introducing fluid from a cowpox pustule into people by inoculation could protect them from smallpox. To test this, he inoculated an eight-year old boy with fluid from a cowpox postule and later infected the boy with smallpox. The boy was immune to small pox as predicted. Louis Pasteur performed similar trials using the bacteria thought to

cause cholera in chickens and demonstrated protection of previously injected chickens after challenge with fresh virulent stock. He coined his attenuated strain a vaccine (from the Latin vacca, meaning ‘’cow’’) in honor of Jenner’s work with cowpox.

Since these pioneering efforts, vaccination has until now become one of the most successful public health initiative ever achieved with the global eradication of childhood diseases such as smallpox and poliomyelitis. Nevertheless, efficient vaccines for several diseases such as tuberculosis (TB), leprosy, and parasitic diseases such as leishmania, malaria and schistosomiasis remain a dream. Efficacious vaccines against human immunodeficiency virus (HIV-1), herpes simplex virus (HSV), dengue, cytomegalovirus (CMV) and several tumours are lacking (Zinkernagel, 2003). Furthermore, the protective capacity of some antiviral vaccines such as those against measles and mumps are in doubts because viral breakthroughs may occur (Wild, 1999). Most vaccines currently in clinical use have been developed using mainly empirical approaches (Lambert and Siegrist, 1997). The protection provided by these vaccines is antibody based, rendering the quality of the response and the antibody avidity the factors determining the efficacy whereas the durability of protection relies to a large extent on B cell memory.

Despite the outstanding progress made in the field of vaccination, there is a serious need for vaccines against several diseases. Infections caused by parasitic agents induce some of the most devastating and prevalent diseases of humans, livestock and companion animals. In fact, malaria is a serious problem globally, responsible for the third most common cause of human mortality (Gardiner et al., 2005). Other similarly devastating parasitic diseases of humans include amoebiasis, filariasis, schistosomiasis, cysticercosis, toxoplasmosis and leishmaniasis, which cause untold morbidity and mortality (Brooker et al., 2006; Schwartz et al., 2006; White and Garcia, 1999). Millions of deaths per annum worldwide are caused by diseases such as malaria, tuberculosis, cancer and HIV-1 for which no effective vaccines exist. It is estimated by world health organization (WHO) that some 16000 people a day or some 5.8 million a year become infected with HIV-1. Furthermore, the WHO estimates that 20%-35% of infant deaths in the world are because of diseases that could be easily prevented if vaccines existed for these ailments.

Therefore the development of alternative and better vaccine strategies that will refine the delivery of specific immunogenic antigens and immunostimulatory vaccine components to the appropriate determinants of an effective immune response with the hope of eliciting a strong primary immune response and a durable immunological memory remains a priority (Zinkernagel, 2003). Despite the wealth of information on microbial pathogenesis and host

defense mechanism currently available, antigenic variation and strain diversity represent only the tip of an iceberg of the complicating factors affecting the vaccinologist.

3.2.1 Today's successful vaccines

Successful vaccines that provide proven protection are vaccines that elicit neutralizing antibody responses of long duration usually after about three boosts. Such vaccines include the smallpox, poliovirus vaccine I, II, III, rubella, tetanus vaccine and bacteria toxins. In contrast, vaccines that do not work satisfactorily or fail to induce long term protection are vaccines against pathogenic infections usually establishing persisting infection in non-lymphoid cells that require a strong CTL response for elimination of the pathogen.

Good examples of unsuccessful vaccines involve some viral infections including human immunodeficiency virus, herpes viruses and hepatitis C virus as well as most of parasitic infections such as malaria, leishmaniasis, schistosomiasis and some bacterial infections like TB and leprosy. Induction of neutralizing antibodies in such infections is not sufficient to eliminate or keep these infections in check, signaling that an effective vaccine against most of such infections will require T cell mediated effector mechanisms in addition to protective antibodies. Interestingly, T cell mediated protection against leprosy or TB mostly depends on constantly activated effector T cells to check reemergence, expansion and spread of the infection (Mackaness, 1969). High levels of protective $CD8^+$ T cell memory depends on persistent infection and T cell activation whereas $CD4^+$ T cell could be maintained by non replicating antigen stored as complexes on follicular dendritic cells or granulomas. Since the endogenous pathway ensuring MHC class I peptide loading generally depends on intracellular synthesis and generation of peptides, many successful vaccines could be attempted employing special tricks (Ochsenbein et al., 2000; Yewdell et al., 1999).

3.2.2 Major challenges of vaccine development

The road to successful development of a vaccine that can be approved for human use manufactured at reasonable cost and efficiently delivered to at-risk populations is costly, long and tedious. Production of material that can be tested in humans and how these tests are performed in clinical trials are tightly regulated. Even those candidate vaccines that survive the initial scrutinization and are approved for use in human trials are not guaranteed to find their way into common usage.

Induction of measurable antibodies by immunization has often been used as a landmark for predicting vaccine efficacy (Roost et al., 1995) and antibody-mediated protection is an essential component of all working vaccines.

The ability for a vaccine antigen to generate protection is a major complex challenge that is beyond the antibody response itself and in particular the development of a global vaccine against any pathogen is made difficult by the degree of variability that exists within the species of pathogen or amongst different serotypes of that pathogen. In fact the success of the first vaccines is because those organisms had little variability. Vaccines against stable diseases such as smallpox, measles and rubella were very effective in conferring protection. Furthermore, vaccines against organisms with a single virulence determinant have been very effective. Additionally, though the genomes of diphtheria causing agent, tetanus and pertussis (Preston et al., 2004) may vary considerably, the major virulence factor in each of these is a single toxin. Thus the neutralization of these toxins by antibodies efficiently prevents disease.

The development of polio vaccine already was much more difficult because it needed three strains of viruses to acquire global coverage, but at least these strains are relatively stable (De Jesus, 2007). Though the degree of variability in influenza virus is quite high between one year and the next, the infection period is also very short making a yearly vaccination with the correct strain very effective (Carrat and Flahault, 2007). However, the capacity of influenza virus to reassort its genome with other strains during co-infection in animal hosts leads to the generation of virulent pandemic strains to which exist no prior immune priming (Hilleman, 2002). Generally, variation in viruses with RNA genomes largely depend on the inaccurate replication since these usually lack proof reading during replication or in the case of influenza virus to genome reassortment, usually driven by immune pressure and selection. More so, viruses such as HIV vary even within a single host during a single infection and therefore have successfully resisted all attempts to date to succumb to a vaccine (Korber et al., 2001).

HCV vaccine development is also marred by rapid mutation particularly in the hot spots within the E2 structural gene which leads to alteration of the immuno-type of the virus even within a single infected host thus it is actually not clear whether the immune response induced by infection with HCV can prevent re-infection. Additional to the genetic variability, HCV (Frazer et al., 2007) as well as EBV (Stanberry et al., 2002) vaccine development have been hindered by the lack of tractable animal models of infection.

On the other hand bacterial antigenic variation accounts for most of the difficulties encountered by vaccinologists in developing vaccines capable of conferring a broad protection, especially in serotypes with very high capsular polysaccharide antigen variability and where

there is very limited cross-protection between serotypes. Pneumococcus, a good example, has over ninety serotypes with little cross-protection (Lipsitch and O'Hagan, 2007). Prophylactic vaccines against uropathogenic *E. Coli* are under study (Stapleton, 2003), but none is in advanced development. Vaccines against nosocomial Gram-negative opportunists remain unlikely because of the diversity of species potentially involved and the fact that many high risk patients are unable to mount a strong immune response (Livermore, 2007).

Parasites often exhibit various immune evasion strategies such as antigenic variation, molecular mimicry and sequestration at both the individual and infective population levels. Additionally, they exhibit complex life cycles and other biological characteristics which pose a real challenge and complicate vaccine development against them (Good et al., 2004). Furthermore, any immunologic memory must be generated such that upon any future re-exposure to the same pathogen, adequate specific antibodies generated will be fast and localized to the site of the pathogen.

Failure of several vaccine strategies could be explained by the lack of precise delivery of their antigenic genetic load into the appropriate immune components capable of driving the much desired response. Dendritic cells possess precise systems (Guermonprez et al., 2002; Trombetta and Mellman, 2005) with several binding receptors capable of delivering antigens to the correct processing compartments (Dudziak et al., 2007; Trombetta and Mellman, 2005). DCs are specialized in presenting peptides from processed antigens to $CD4^+$ and $CD8^+$ T cells, self and microbial glycolipids to NKT cells and native antigens to B cells. Therefore efficient targeting of these specialized immune components is a crucial milestone in efficacious vaccine development. In addition to targeting the antigenic material to DCs, these cells require the concomitant delivery of adequate activation-maturation signals that enables them to migrate, process and deliver the antigenic components at the correct address for subsequent action. Some viruses such as influenza (Cella et al., 1999) can promote the maturation of DCs and still other viruses may fail to do so though engineering of these viruses to utilise or express receptors that promote maturation of DCs has been attempted with some success (Molinier-Frenkel et al., 2003; Okada et al., 2001).

In the case of viruses that exist in multiple strains or can rapidly undergo mutation due to immunologic pressure, either the antibodies must be targeted against a conserved epitope shared between strains or unlikely to mutate or the vaccine must include antigens from each relevant strain. Vaccine strategies based on live or inactivated viruses, attenuated or vectored viruses which may naturally have the tropism to various immune components may be ideal candidates since they have evolved the natural mechanism to enter these cells. Compounded by the risk of

reversion to wild type because of co-infection or incomplete inactivation, live or inactivated viruses will be neglected in preference to vaccines based on viral vectors. An essential property of viral vectors is the ability to non-specifically activate the immune system (Truckenmiller and Norbury, 2004). Thus by triggering pattern recognition receptors such as those of the TLR family (Akira, 2004; Beutler et al., 2003; Peiser et al., 2002) or the scavenger receptor family (Peiser et al., 2002), the efficiency of antigen presentation can be improved so that the efficiency to generate potent $CD8^+$ T cells can be increased (Schwarz et al., 2003). Furthermore, alphavirus vector has been reported to break tolerance by the induction of signalling through protein kinase receptors that led to a productive $CD8^+$ T cell response (Leitner et al., 2003).

Since adjuvant may be used to increase or fine tune the immune response to an antigen, the particular adjuvant choice may make a difference in a particular vaccine formulation depending on the desired outcome. Despite the evaluation of a variety of adjuvant, aluminium salts are currently the only adjuvants in mass usage. The function of aluminium salts originally thought to form a depot of the antigen has been recently questioned (Flarend et al., 1997). However, since the use of aluminium salts as adjuvant has been predominantly involved in the induction of antibody responses, the discovery of new adjuvants for the development of vaccines necessitating cell-mediated immune response is crucial (Hunter, 2002; Ulmer et al., 2006). The heightened stringency on vaccine safety has plunged new vaccines into a situation that is often linked to lower immunogenicity compared to previous whole cell or virus-based vaccines. This situation calls for the necessity to employ adjuvant to induce potent and durable immune responses with additional benefit that less antigens and fewer injections may be needed (Guy, 2007). Currently, there is an evaluation spree for cytokines as adjuvant in vaccines as against most chemical entities because cytokines are likely to represent the direct proximal mediators of the ‘classical’ adjuvant (Pashine et al., 2005).

Therefore, one challenge is to chemically develop adjuvant or code in natural adjuvant that would be effective, yet do not induce extremely vigorous immune response either systemically or at the local injection site, causing sequelae such as granuloma formation (Cox and Coulter, 1997; Gupta and Siber, 1995). The best would be adjuvants that are effective and can be used either singly or in combination in promising upcoming vaccine strategies such as in the multigene RNA vaccine vector strategy (Thiel et al., 2003) which could in principle represent the design of a ‘perfect mix’(Guy, 2007), an optimal, safe combination of cytokines that will not only yield an additive but a synergistic effect, to eventually drive the desired immune response.

Indeed, a major pitfall to the success of most vaccines in chronic infections as well as cancer vaccines might be the presence of regulatory/suppressor T cells (Sakaguchi, 2000;

Shevach, 2000). In fact a huge amount of experimental evidence implicate these T cells for suppressing anti-tumour immunity and pointing out that the removal of these cells leads to tumour clearance (Steitz et al., 2001; Sutmuller et al., 2001). Furthermore, more recent reports suggest the involvement of these cells in restricting memory $CD8^+$ T cell responses (Kursar et al., 2002; Li and Wu, 2008; Todryk et al., 2008). Additionally, an increased frequency of $CD4^+$ $CD25^+$ T cells has been observed in the blood and tissue of patients with cancer (Viguier et al., 2004; Wang et al., 2004; Woo et al., 2001). Distinct DC maturation stimuli will have different capacities to induce such regulatory T cells therefore, a carefully designed vaccine incorporating components that will dampen such regulatory T cell effects might be instrumental to the efficacy of such a vaccine.

The safety of all vaccines must be a priority most especially if the vaccine is intended for human use. The attenuated live measles vaccine (Edmonston strain) approved in the early 1960s was used for over a decade though it caused a high incidence (20%-40%) of fever unless it was administered together with immunoglobulins. However, an inactivated version of this same vaccine that was also administered in conjunction with a live vaccine was reported to be less effective despite avoiding the side effects of the live vaccine. Vaccinees not only showed immunity of shorter duration but were at risk for atypical measles infection (Lampe et al., 1985). Thus an efficacious vaccine must provide the necessary protection and still be very safe.

3.3 Viral vectors as vaccines

Viruses have evolved highly efficient mechanism to get into cells and thus utilizing the cellular machinery for the production of virally encoded proteins. Therefore, viral vectors are naturally preferred vehicles for heterologous gene delivery into cellular components of the immune system for induction of an optimal immune response. Some viruses specifically interact with receptors or some carbohydrate parts on the surface of APCs and directly infect them, though this is not a guarantee for an effective vaccine or immunotherapeutic vector. This is because it is well known that humoral responses can develop even if antigen is not made by an APC but through the mechanism of cross presentation whereby a cell expressing an antigen can transfer the antigen to professional antigen presenting cells for the generation of MHC class I-restricted cytolytic T lymphocyte responses (Gauvrit et al., 2008; Howland et al., 2008).

During the past few years, several vectors tailored to efficiently carry and deliver genes for immunization have been developed with the added advantage that infections by these natural

immunogens will mimic real life offering at least the possibility of tickling both arms of the immune system (Esposito and Murphy, 1989; Murphy, 1989; Taylor and Paoletti, 1988). The failed efforts to develop effective vaccines against HIV-AIDS and malaria led to an explosive development of a myriad of innovative viral vectors capable of delivering antigens and induce immune responses (Girard et al., 2006; Gluck et al., 2005; Sutter and Staib, 2003). Safety and immunogenicity an important recurrent issue for the development of live-attenuated vaccines demands a striking balance between both. Thus, most replication competent viral vectors have not been considered sufficiently safe to enter human clinical trials, inspiring the development of several DNA and RNA viral vaccine vectors engineered to be replication-incompetent (Oertli et al., 1996; Schindler et al., 1994; Warden and Weir, 1996). Furthermore, engineered vectors originating from non-human viruses with low prevalence within the human population or from viruses originating from discontinued vaccines may be considered attractive since they have almost no pre-existing immunity in humans. However, it is not clear whether pre-existing immunity could be generalized to all vectors. It was demonstrated earlier that pre-existing antibodies to measles virus did not preclude induction of cellular and humoral immunity after revaccination (Zuniga et al., 2007). In addition to proven efficacy and safety, a prerequisite for every vaccine is the feasibility to be manufactured in large scale while maintaining the genetic stability of the vaccine vector over several viral generations even after vaccine administration and residual propagation in vaccinees. It should be emphasized here that each specific technological platform exhibits some advantageous features and potential limitations depending on the intended application.

3.3.1 Adenovirus vectors

Adenoviruses are DNA viruses that cause diseases ranging from subclinical infections to diseases of multiple organ systems particularly in immune compromised individuals. These viruses are generally quite species-specific offering a complication for preclinical studies in humans since many people have been previously infected with adenoviruses especially during infancy. Adenoviruses have been exploited as vectors for gene delivery either as vaccine or gene therapy because of their extensive tropism and their growth kinetics (Danthinne and Imperiale, 2000). They get into target cells by the use of the coxsackie/adenovirus receptor (Bergelson et al., 1997) or by internalization after binding to cellular integrins notably αvβ5 (Wickham et al., 1993).

Adenovirus vectors can be of different flavours notably, either replication-incompetent due to the complete deletion of E1 or E1 and E3 and complemented in trans in cell culture or replication-competent. The replication competent vectors can afford a cloning capacity of 3-4 kilo bases (kb) while the replication-incompetent counterparts can afford a trans-gene capacity of 7-8 kb. The cloning capacity for heterologous genes can be increased depending upon how many original early regions are deleted. There exist some safety concerns with adeno-vectors that relate to the inflammatory response generated by the vectors. Additionally, there are disturbing concerns about possible integration even though the wild type virus has been reported not to integrate into the genome of the infected cells (Harui et al., 1999).

The most explored for vaccine development is the serotype 5 but has been reported to have the highest seroprevalence in the world (Shiver and Emini, 2004). Therefore adenoviruses from poorly circulated strains or even from chimpanzee provide an alternative as vaccine and gene therapy vectors.

To avoid pre-existing immunity and the possibility of hampering vaccine efficacy, novel strategies are being developed utilizing altered surface proteins or chimeric virus carrying the surface proteins of another serotype (Nanda et al., 2005; Roberts et al., 2006; Thorner et al., 2006; Xin et al., 2005). Adenovectors have been developed and tested for ailments ranging from infectious diseases to cancer for both animal and humans.

The first licensed gene therapy product, Genedicine (Peng, 2005) approved in China in 2003 is based upon an adenovector encoding tumor suppressor p53 for use in the treatment of squamous cell head and neck cancer. Vaccines based on Adenoviruses are being developed for malaria (Li et al., 2007), SARS (Chunling et al., 2006; Zhi et al., 2006) and Ebola (Sullivan et al., 2003). Furthermore, a nasal application of an adenovirus vector expressing an influenza gene has demonstrated safety and immunogenicity in humans. Adenovectors are also being investigated as vaccines for animal diseases such as rabies (Culp et al., 2006) and foot and mouth disease (Liu et al., 2006).

3.3.2 Adeno-Associated Virus (AAVs)

Adeno-associated viruses (AAVs) are non-enveloped, single stranded DNA viruses with a genome size of 4.7 kb belonging to the *parvoviridae* family that depend on helper virus functions for replication. Although AAV has so far not been linked to any human disease, there are 11 serotypes (from humans to primates) defined by AAV-specific antibodies (preferentially to AAV-2) which have been detected in 70% - 80% of the human population (Romano, 2005).

Recombinant AAV vectors (rAAVs) are capable of inducing trans-gene expression in a broad range of tissues. Unlike the wild type AAV, rAAVs may insert their genome into the safe AAVS1 site of the host cell chromosomes owing to deletion of the rep gene. However, rAAVs may insert their DNA randomly into the host-cell genome and thereby silence tumor-suppressor genes (Romano, 2003, 2006). The genomes of rAAVs are basically composed of an expression cassette, with a limited insertional capacity of 5 kb, flanked by the inverted terminal repeats taken from wild type viruses. Therefore, production of viral particles is dependent on cells that contribute the Rep, Cap and virus helper function in trans.

In fact, the generation of vaccine candidates acceptable for human clinical applications involves extensive purification of rAAV particles using the method of chromatography (Merten et al., 2005; Morenweiser, 2005). Intramuscularly injected rAAV expressing SIV antigens in pre-clinical studies demonstrated protective, persistent antibody and T-cell responses towards SIV antigens (Johnson et al., 2005). Interestingly, rAAV particle vaccine candidates geared against AIDS have been developed using targeted genetics. These vaccines have been reported to have passed Phase I trials in Europe, India and are currently undergoing Phase II evaluation in Africa (Girard et al., 2006).

3.3.3 Vesicular stomatitis virus

Vesicular stomatitis virus (VSV) is an arthropod borne rhabdovirus (a family also containing rabies virus) with a single stranded negative-sense RNA genome of about 11kb that normally infects domestic animals causing a rarely fatal disease. It exist in several serotypes and in humans the virus causes mild and self-limiting flu-like symptoms although more serious conditions such as encephalitis may occur (Quiroz et al., 1988). Generally livestock infections are associated with either serotype VSV Indiana or VSV-New Jersey.

VSV was rescued from cDNA like measles Virus using reverse genetics. The genome can be engineered to encode heterologous antigens and recombinant VSV vectors grow to high titers and are genetically quite stable. They have a replication strategy that is strictly limited to the cytoplasm of the infected cell, precluding any risk of viral derived nucleic acid recombining with the host cell chromosomal DNA. An HIV-vaccine based on live-attenuated VSV vector expressing HIV *env* and *gag* genes was successfully tested in the simian/human immunodeficiency virus (SHIV)/macaque model. Since VSV is among the neurotropic viruses, serious safety concerns are considered for a mucosal application of rVSV in human clinical trials. Intranasal vaccination of young mice with the vectors did induce encephalitis (Reiss et al.,

1998) which provoked the development of a new generation of vectors by reshuffling viral genes within the VSV genome or by truncating the cytoplasmic tail of VSV encoded G protein. VSV vectors devoid of the entire G gene were generated in cell lines providing the G protein in trans (Publicover et al., 2005). Amazingly, these single-cycle vectors expressing HIV-*env* induced primary and memory $CD8^+$ T cell responses that were comparable to replication-competent rVSV. The expression of VSV G is toxic to most mammalian cells which may limit the development of complementing cell lines at the industrial level (Chen et al., 1996).

3.3.4 Measles virus

Measles virus (MV), a negative, single-stranded RNA virus with a genome size of 15894 nucleotides is being exploited as vaccine vectors in the HIV-1 vaccine arena. In fact, several recombinants encoding several other important viral genes have been constructed, including immunogenic genes of hepatitis B, mumps, West Nile virus, and severe acute respiratory syndrome coronavirus (SARS) (Liniger et al., 2007).

The measles virus as vaccine vector has several qualities making it attractive for HIV/AIDS vaccine. Experience with the live-attenuated measles vaccine demonstrate the possibility to induce life-long immunity composed of cellular and humoral immunity that persist for up to 25 years (Zuniga et al., 2007). The ability of measles virus to infect dendritic cells and macrophages may be responsible for this property. The virus replicates in the cytoplasm, therefore precluding integration into the host cell genome. The helical structure of the nucleocapsid permits the vector to accommodate up to 5 kb of heterologous gene insert. Additionally, a high stability after several passages has been demonstrated for this virus in vitro (Zuniga et al., 2007). Interestingly, it was also shown that SIV/HIV antigens do not incorporate into measles virus virions and therefore did not alter their tropism (Zuniga et al., 2007). About 90% of the human population has been in contact with measles or its vaccine during infancy. Despite this exposure, the presence of antibody and cellular immunity seem not to significantly inhibit a booster vaccination 10 years after the primary infection or vaccination (Brave et al., 2007). A measles virus recombinant encoding the HIV envelope glycoprotein elicited high titer antibodies and HIV envelop specific $CD8^+$ and $CD4^+$ cells following a single injection. The antibodies were able to neutralize the HIV neutralizing strain as well as several other heterologous isolates (Lorin et al., 2004). Similar results have been reported after vaccination of humanized mice for MHC class I HLA-A0201 (Lorin et al., 2005). In fact, the quality and magnitude of antibodies induced by recombinant measles vaccines were comparable to those of

the conventional measles vaccine. However, it remains to be seen whether the replacement of the current measles components of the globally accepted MMR regimen is feasible from a regulatory and licensing stand point (Liniger et al., 2007).

3.3.5 Herpes virus

Herpes simplex virus (HSV)-1 a highly pathogenic virus infecting a variety of tissue types but also targeting mucosal epithelial surfaces. Lysis of infected cells leads to infection of sensory neurons during which the virus typically enters into a lytic or a latent phase and in the worst scenario may cause encephalitis. This double-stranded DNA virus with a genome of 152 kb contains 81 genes encoding for at least 84 polypeptides (Marconi et al., 1996; Marconi et al., 1999).

After deletion of the non-essential genes, HSV-1 offers a cloning capacity of about 50 kb. Currently, two basic approaches for the development of HSV-based vaccine vectors include amplicons and recombinant viruses. Amplicons work by transfection of plasmid DNA into a cell line equally transfected with a helper virus providing the necessary structural and regulatory proteins in trans (Spaete and Frenkel, 1982). HSV-1 amplicon expressing HIV-1 gp120 induced cellular and humoral immune responses in immunized mice (Hocknell et al., 2002). Replication-deficient vaccine vectors based on HSV-1 have been constructed as vaccine vectors against HIV-1.

These replication deficient vaccines expressing *Env*, *Gag* and a *Tat-Rev-Nef* fusion protein of SIV were able to induce robust anti-*Gag* and anti-*Env* cellular immune responses in macaques (Kaur et al., 2007). A limitation in the use of HSV-1-based vectors could be the high pre-existing immunity within the human population. Furthermore, mass vaccine production may be difficult because of technical difficulties to achieve high titers (Dudek and Knipe, 2006).

3.3.6 Poxviruses

The excellent success of vaccinia virus in the eradication of smallpox increased the interest to exploit the poxvirus as vaccine vectors. Poxviruses are double stranded DNA viruses with a genome of 150-300 kb, replicating in the cytoplasm. The first live recombinant vaccines developed were based on vaccinia virus as a vector (Arlen et al., 2005; Mastrangelo et al., 2000). The advantage is that Poxvirus vectors easily grow to high titers and are genetically easy to manipulate. However, because of safety concerns on the use of replicating vectors in immune

suppressed individuals, safer non-replicating vectors have been at the center of recent developments. These include the modified vaccinia Ankara (MVA) which due to serial passages in chicken embryo fibroblast has lost about 15% of its genome, and NYVAC, derived from the Copenhagen strain of vaccinia made replication incompetent by 18 specific deletions (Parrino and Graham, 2006). Fowl-pox (FPV) and canary-pox virus (ALVAC) vectors that usually do not replicate in humans are now undergoing some clinical trials. The FPV and ALVAC will certainly not face the limitation of pre-existing immunity. In clinical trials ALVAC-HIV recombinants demonstrated safety but induced only modest HIV-specific cellular immune responses. In fact, a phase II study of a multi-gene ALVAC-HIV vaccine candidate failed to elicit a $CD8^+$ CTL frequency of 30% in healthy volunteers despite a boost with recombinant gp120 (Russell et al., 2007). Despite the moderate immunogenicity profile recorded in clinical trials with recombinant poxvirus-based vaccines, these vectors may only be attractive as booster components in heterologous prime-boost regimens (Girard et al., 2006; Naslund et al., 2007; Robinson, 2002).

3.3.7 Alphavirus vectors

Alphaviruses are positive sense single stranded RNA viruses with a genome size of approximately 12 kb. They include Semliki Forest (SFV), Venezuelan equine encephalitis virus (VEEV) and Sindbis virus (SIN) belonging to the *Togaviridae* family. The high interest in the development of Alphavirus-based vaccine vectors stems from the attractive intrinsic characteristics such as their replication which is limited to the cytoplasm, therefore excluding any integration of virus derived sequences into the host cell genome, their ability to infect a wide variety of cells including mammalian cell types and the capability to transiently express high amounts of viral proteins or heterologous proteins. Furthermore, the complete lack of antivector immunity in the human population heightens their attractiveness (Schlesinger and Dubensky, 1999; Strauss and Strauss, 1994).

Engineered Alphavirus-derived replicon vaccines have been developed by replacing viral genes with heterologous genes. The formation of virus-like particles (VLPS) necessitated a packaging cell system through which the necessary structural proteins required for virus particle formation were provided in trans (Polo et al., 1999). Indeed attractive for the vaccinologist, these vectors retained their capacity to express genes and replicate in cells but completely lost the ability to infect neighboring cells. This led to the coinage of single-cycle vectors (Liljestrom and Garoff, 1991; Tubulekas et al., 1997). Furthermore, particles derived from these vectors have

been directly implicated to interact efficiently with antigen-presenting cells such as dendritic cells (Gardner et al., 2000).

VEEV-SIV replicon particles demonstrated protective immune responses against simian immunodeficiency virus (SIV) in primates (Davis et al., 2000; Perri et al., 2003). Clinical experimental trials with the VEEV replicon particles will be of high interest to demonstrate the potential of these particle vaccines encoding a clade C HIV-1-*gag* gene (Alphavax). These vectors may encounter serious regulatory difficulties at the level of obtaining a cGMP-grade manufacturing cell line that will enable mass production of the vaccine for commercial purposes.

3.4 Coronavirus biology and their exploitation as vaccine vectors

The entry of coronaviruses into susceptible cells is implicated to the spike glycoprotein peplomers on the membrane of the virion. The spike is responsible for ligating into specific receptors on susceptible cells leading to a conformational change which subsequently leads to the fusion of the two membranes liberating the viral genome into the cytoplasm. Coronaviruses are enveloped, positive-stranded RNA viruses with a size of approximately 30 kb and possess the ability to replicate autonomously in susceptible target cells. More than two third of the genome encodes the replicase gene while the remaining one third encodes mostly the structural and the nonessential genes depicted in figure 3 below. The replicase gene is composed of two large open reading frames (ORFs), ORF1a and ORF1b which can be translated into two polyprotein (pp) precursors, ppIa and ppIab. Translation of the complete replicase polyprotein ppIab necessitates a (-1) ribosomal frame shift. Extensive processing of this large polyprotein by virus-encoded proteinases leads to the production of viral proteins which together with some undetermined cellular components assemble to form an active replicase-transcriptase complex.

In addition to replicating the genomic RNA, this unique transcription machinery produces a nested set of six to eight sub-genomic mRNAs (sgmRNA). These sgmRNAs are produced in constant but nonequimolar ratios and appear structurally polycistronic though they are functionally monocistronic.

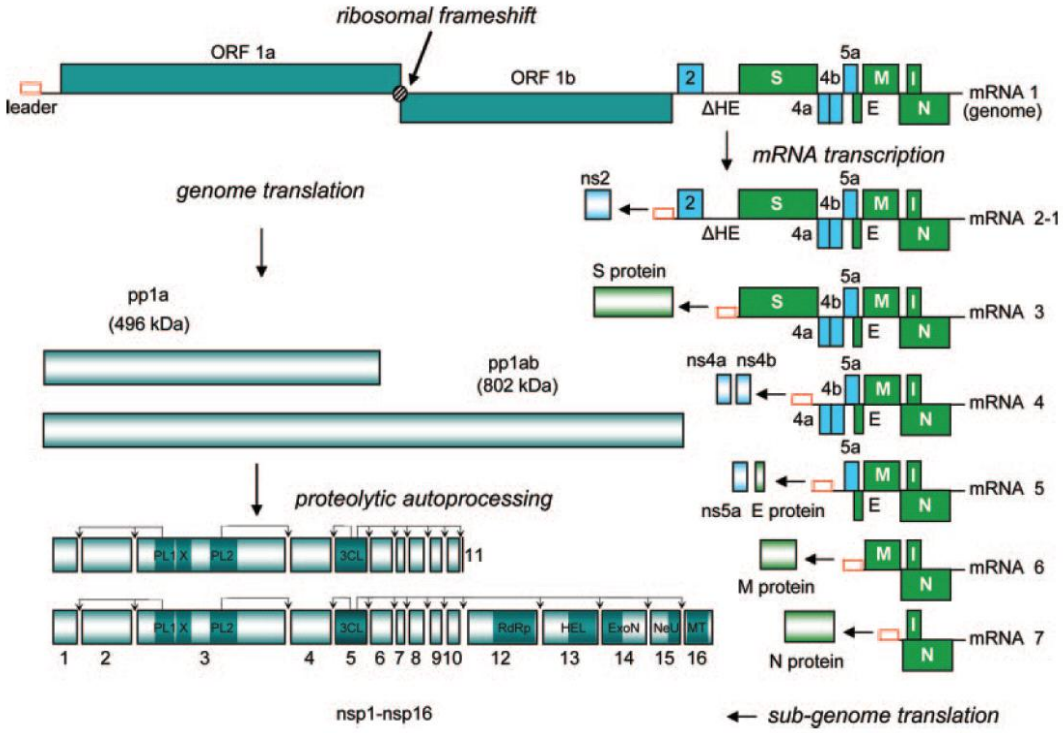

Figure 3: Genome organization and expression of the MHV-A59 prototype virus. The structural relationships of the MHV-A59 genome and the subgenome length mRNAs are depicted. The virus ORFs are shown in teal (nsp1-nsp16 genes), blue (ns2, ns4a, ns4b, and ns5a genes), and green (S, E, M, N and I structural genes). The ORFs are defined by the genomic sequence of MHV-A59 (Coley et al., 2005). The open red box represents the common 59-nucleotide leader sequence and the barred circle represent the programmed (-1) frame shifting element. The translation products of the genome and subgenome length mRNAs are depicted and the autoproteolytic processing of the ORF 1a and ORF 1a/ORF1b (Sawicki et al., 2007).

This therefore generates a gradient-like synthesis of the subgenomic mRNAs with the highest amount closest to the 3' terminus of the genome (Hofmann et al., 1993; Konings et al., 1988; Sethna et al., 1989) with exceptions to transmissible gastroenteritis virus (TGEV) and the feline infectious peritonitis virus (FIPV) where the smallest sgmRNA is produced in much smaller amounts than the next smallest (de Groot et al., 1987; Sethna et al., 1989). Most of the structural genes and the nonessential genes can be replaced by genes encoding antigens and immunostimulatory molecules. Therefore, this specialized transcription machinery enables a multi-heterologous protein expression amenable to exploitation for multi-gene vaccine development. Furthermore, one important advantage is that, coronaviruses have the largest known positive-stranded RNA genome and thus can afford a cloning capacity of 6-9 kb of heterologous genes though no one has actually attempted demonstrating the limits. Additionally,

it is possible to steer these viruses to target specific immune components by manipulating the spike glycoprotein implicated for tropism.

Currently, several coronaviruses of medical and veterinary importance have been characterised. They infect various animal species, causing respiratory, gastrointestinal, cardiovascular and neurological ailments. In humans, coronaviruses have been associated mostly with common cold, diarrhoea and most recently the severe acute respiratory syndrome (SARS) epidemic which highlighted the importance of coronaviruses.

Despite the enormous intrinsic advantages and promising qualities that coronaviruses possess, not much has been exploited of this ‘’Gold mine’’. Therefore not much is known on coronaviruses as vectors for vaccines or immunotherapy probably because of the former technical limitations to conduct effective mutagenesis on these large and complex RNA genomes. Furthermore, almost nothing is known about the immunogenicity of coronavirus-based vectors and their characteristics.

3.4.1 Immunobiology of coronavirus infection

Coronaviruses infect several animals including humans and induce diseases ranging from mild common cold, enteric infections, hepatitis, demyelinating disease of the central nervous system (CNS) (Weiner, 1973) to acute and chronic respiratory diseases. Some of these ailments have engraved serious economic losses and even death in the human population (Stadler et al., 2003). While little is known about the pathogenesis of any of the human coronaviruses (229E, OC43, HKU1, NL63, and SARS-CoV), there have been detailed studies of the pathogenesis of some animal coronaviruses. Some examples of these include the porcine coronavirus, avian coronavirus, feline coronavirus, bovine coronavirus and the murine coronavirus (Drosten et al., 2003). Murine coronavirus (MHV) is the most studied of all coronaviruses, with several strains exhibiting differences in tropism and levels of virulence. The commonly used laboratory strains infect primarily the liver and the brain providing excellent models for encephalitis and hepatitis as well as the immune-mediated demyelinating disease (Perlman, 1998). MHV infection is regarded as one of the best animal models for the study of demyelinating disease such as multiple sclerosis (Barthold et al., 1993).

Compared to other models of infection and virus-induced encephalitis (Dorries, 2001; Griffin, 2003), intranasal or direct intracranial MHV infection induces a vigorous CNS inflammatory response composed of both innate and adaptive immune components (Bergmann et

al., 2003; Zhou et al., 2003). Furthermore the part played by the immune response to MHV infection in viral clearance and pathogenesis in the CNS has been analyzed (Koren et al., 2003).

To protect against coronavirus infections both cell-mediated and antibody immune responses are needed. During the acute phase of the infection $CD4^+$ and $CD8^+$ T cells are required for clearance of the virus (Beauchemin et al., 1999; Belay et al., 2005; Bergmann et al., 1993) mainly utilizing perforin-mediated killing from astrocytes and microglia while IFN-γ has been implicated in virus clearance from oligodendrocytes (Parra et al., 1999). Additionally, most $CD8^+$ T cells and $CD4^+$ T cells during the height of T cell accumulation within the CNS are virus specific (Bergmann et al., 1999; Marten et al., 2001). The accumulation of virus specific $CD8^+$ T cells in the CNS is 10-fold more than in the periphery and are of the phenotype $CD44^{hi}$, $CD62L^{-/Lo}$, $CD11a^{hi}$ and CD49d consistent with their function in controlling acute MHV replication (Bergmann et al., 1999; Marten et al., 2003).

Mapping of MHV T cell epitopes have been localized to several structural proteins though there might be additional epitopes in the two third part of the genome encoding the replicase gene products that may warrant examination. The spike of MHV which is implicated in virus tissue tropism and is responsible for cell-to-cell fusion during infection has been identified with $CD8^+$ T cell epitopes. Within the MHV spike is located an immunodominant $CD8^+$ T cell epitope (S510-S518) and a subdominant $CD8^+$ T cell epitope (S598-S605) in C57BL/6 mice. Furthermore, in BALB/c mice, MHV nucleocapsid protein is associated with a $CD8^+$ T cell epitope (N318-N326) (Bergmann et al., 1993). Additionally, $CD4^+$ T cell epitopes have been mapped in the spike (van der Veen, 1996), membrane protein (M) (Xue et al., 1995) and the nucleocapsid protein (N) (van der Veen, 1996). Interestingly, B-cell epitopes essential for neutralizing antibody formation have been mapped as well in the spike protein while non neutralizing antibodies have been identified in other structural proteins (Daniel et al., 1993; Daniel et al., 1994; Talbot and Buchmeier, 1985). The clearance of MHV in the CNS is primarily executed by cell-mediated immune response while antibodies may be important in preventing re-emergence of the virus.

Infection with the neurovirulent JHM strain is identified with a strong and durable IFN-α/β response with high levels of chemo-attractants such as CCL3 (MIP-Iα), CCL4 (MIP-Iβ) and CXCL2 (MIP-2) as well as CXCL10 and CXCL5 (RANTES) (Laude et al., 1992). This increase in chemokine secretion correlates with high numbers of macrophages, neutrophils and natural killer (NK) cells during the acute phase of the infection and also in the later stages of the demyelinating disease (Glass et al., 2002). Studies with recombinant viruses implicate the

infiltration of macrophages to be a likely influence of the spike protein (MacNamara et al., 2005).

Infection of the CNS with other neurotropic viruses such as lymphocytic choriomeningitis virus (LCMV) and measles virus induces a similar chemokine-gene-expression profile to MHV, meaning that CNS resident cells may respond to viral infection in a similar fashion, which could be linked to their expression of the type-1interferons (Asensio and Campbell, 1997; Salazar-Mather et al., 2002).

3.4.2 First steps towards a coronavirus-based vaccine

Coronaviruses are the largest enveloped, positive-stranded RNA viruses known to date, with a genome size of approximately 30 kb and belonging to the family *Coronaviridae* in the order Nidovirales. Their genome is 5' capped, 3' polyadenylated and infectious (Lai and Cavanagh, 1997) upon transfection into target cells. The genome organization is such that the essential genes are arranged in the order 5'-replicase-S-M-E-N-3' and has interspersed between the structural genes, a varying number of non-structural or group-specific genes. These group-specific genes were shown not to be essential at least in tissue culture for the group I coronavirus feline infectious peritonitis virus and for the group II coronavirus mouse hepatitis virus (MHV) (de Haan et al., 2002a).

Coronaviruses are endowed with natural characteristics which make them attractive as vaccine candidates. The extraordinary large genome size indicates that these viruses may have a large cloning capacity and in addition their unique transcription mechanism which allows for the production of 6-8 sub-genomic mRNAs, implicating these viruses as promising candidates for multi-gene RNA vectors (Enjuanes et al., 2001; Thiel et al., 2003). The fact that the accessory genes are dispensable for viral replication ascertains the provision of space for cloning large heterologous genes which can be typically inserted downstream of the so called coronavirus transcription regulatory sequences (TRS) (Curtis et al., 2002; Enjuanes et al., 2001; Fischer et al., 1997; Thiel et al., 2003). The core consensus TRS sequence for MHV has been mapped to the 9-nucleotide motif 5'-AAUCUAAAC-3' (Lai and Cavanagh, 1997). Additionally the deletion of the nonessential or group-specific genes of coronaviruses has been reported to be attenuating in the natural host (de Haan et al., 2002a).

Furthermore, the species and tissue tropism of coronaviruses can be easily modified by manipulating the spike protein which is responsible for targeting. In fact, by exchanging the

ectodomain of the MHV spike with that for FIPV, the murine virus was retargeted to feline cells losing its ability to infect murine cells (Haijema et al., 2003; Kuo et al., 2000) and the reverse was demonstrated for FIPV (Haijema et al., 2003). Similar ectodomain exchanges of the S protein led to changes in tissue tropism of MHV and transmissible gastroenteritis virus (TGEV) respectively (Enjuanes et al., 2001; Navas et al., 2001; Phillips et al., 1999). The deliberate rearrangement of the conserved order of the coronavirus genes has been demonstrated (de Haan et al., 2002b) and this further improves safety of these viruses as vaccine vector candidates. Furthermore, since the replication of coronaviruses is restricted to the cytoplasm, this precludes any risk of integration of virus derived sequences into the host cell genome.

The development of coronaviruses as potential vectors for vaccine or immunotherapy had been perishing in the depths of all potential viruses thought to be of interest as vector candidates due to technical impediments. The extremely large size of the coronavirus genome and more especially the instability of plasmids carrying coronavirus replicase genes hampered the construction of a full length cDNA clone (Masters, 1999). Lately, there has been an explosive demonstration of technical improvement, following reports of successful construction of full length infectious coronavirus cDNA clones (Almazan et al., 2000; Thiel et al., 2001a; Yount et al., 2000) providing an invaluable tool to study coronavirus virus-host interaction, replication, transcription and the exploitation for possible vector generation. Not long after this demonstration of a reverse-genetic system for coronaviruses, were reports of the first coronavirus-based vectors described and coined to be replication-competent but propagation deficient (Curtis et al., 2002; Ortego et al., 2002) based on transmissible gastroenteritis virus (TGEV). The rational of this idea was to generate coronavirus replicons by deleting one or more structural gene(s) and to introduce a system for the production of virus-like particle (VLPs) by complementing the structural gene(s) in producer cells in trans. This was elegantly demonstrated by two independent groups who reported the production of a recombinant TGEV lacking the small envelope protein gene E (ΔE-TGEV) (Curtis et al., 2002; Ortego et al., 2002). Both groups reported that efficient propagation of TGEV vectors lacking the E gene was dependent on E expression in trans, demonstrating the absolute dependence of TGEV virus on the presence of TGEV-E protein for efficient propagation.

Similar observations were recorded for MHV ΔE though this study pointed that MHV E was not absolutely important for virus replication (Kuo and Masters, 2003). Nonetheless, ΔE vectors can be considered to be safe since recombinant MHV devoid of the E gene replicates only to reduced titers. Additionally, despite the provision of TGEV E or MHV E in trans in order to

efficiently propagate these vectors, there has been no evidence of recombination with the E provided by the packaging system, leading to wild type reversion.

To further step up the safety of these vectors, it is possible to rearrange the structural genes (de Haan et al., 2002b) or by using a strategy which allows a reconnection of the transcription circuit (Yount et al., 2006). Noteworthy, is the fact that the group-specific genes have been earmarked as not essential for replication at least in tissue culture and that their deletion is attenuating in the natural host (de Haan et al., 2002a; Haijema et al., 2004), with implication that safety of coronavirus vectors can be further improved by deleting all the group-specific genes.

A multigene vector construct was demonstrated (Thiel et al., 2003) in which three cytoplasmic reporter genes namely chloramphenicol-acetyltransferase (CAT) gene, firefly luciferase (LUC) and green fluorescent protein (GFP) were encoded by a coronavirus-based vector. Furthermore, vector-mediated expression of these genes was shown both at the RNA level and by protein assay with the respective reporter protein assaying techniques (Thiel et al., 2003) . A helper virus was employed in this situation to provide the necessary structural proteins for packaging of the RNA into VLPs, providing a potential source of VLP stock contamination.

A critical consideration for viral vaccine vector is the potential for efficient delivery of the encoded genetic cargo to specific target cells of the immune system. An attractive concept is to deliver vaccine antigens to antigen presenting cells such as DCs through binding of specific receptors on DCs along with stimuli that would influence DC maturation since DCs control a spectrum of the innate and adaptive immune responses (Steinman and Banchereau, 2007). Remarkably, the receptor for human coronavirus-229E (HCoV-229E), human amino-peptidase-N (h-APN or CD13) and for MHV, carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 1 are expressed at high levels on human and mouse dendritic cells, respectively. Furthermore, using HCoV-229E based multigene VLPs it was possible to efficiently transduce mature and immature DCs as demonstrated by VLP-mediated heterologous gene expression (Thiel et al., 2003). Additionally, MHV has been implicated for targeting and infecting murine DCs by several groups (Cervantes-Barragan et al., 2007; Zhou and Perlman, 2006).

The concept of coronavirus-based vectors has been adapted to the murine system. In fact, MHV-based vector RNAs lacking the structural genes E and M have been produced and propagated using E-M complementing packaging cells (Eriksson et al., 2006a). Interestingly, VLPs containing MHV-based vector RNAs were used to transduce murine DCs and 24hrs later vector mediated expression of GFP in those DCs was evident. The murine model of coronavirus-based vectors represent a very important tool for the development and evaluation of the system

and may be instructive for the development, evaluation and adaptation of coronavirus-based vaccine vectors for human ailments (Eriksson et al., 2006b).

3.4.3 Attenuation of coronavirus-based vectors (ΔNspI, ΔNS2, ΔHE, Δgene4/5a and ΔE)

3.4.3.1 Non-structural protein 1 (NspI)

Mouse hepatitis coronavirus replicase encoded gene products when accurately processed yield 16 mature non-structural proteins (Nsp). The expression of nsp1 the N-terminal most gene 1 protein has been implicated to have a pathogenic role in MHV infection (Zust et al., 2007), SARS (Kamitani et al., 2006; Wathelet et al., 2007). Nsp1 was generally found to inhibit host protein synthesis by promoting host mRNA degradation (Kamitani et al., 2006; Zust et al., 2007) and inhibiting translation (Narayanan et al., 2008). Additionally, Rotavirus nsp1 inhibits expression of type 1 interferon by antagonizing the function of interferon regulatory factor -3, 5 and 7 (Barro and Patton, 2007) indicating that this might be a general mechanism to interfere with the first wave of immune response to the advantage of the virus. Amazingly, nsp1 can be partially deleted without perturbing viral replication in tissue culture (Brockway and Denison, 2005). Furthermore, the deletion of 99-nucleotides from MHV-A59 nsp1 resulted in a mutant (MHV-Δ99) which grew to comparable wild type MHV-A59 titers in tissue culture. Interestingly, like the wild type MHV-A59, this nsp1 mutant virus still retained its ability to infect professional antigen presenting cells. MHV-A59 is hepatotropic and is known to cause hepatitis in the natural host, nonetheless, when a high dose, 5000 pfu of the MHV-nsp1 mutant was used to infect mice, the mutant demonstrated compounding attenuation in the murine host as compared to the wild type (Zust et al., 2007). The MHV-nsp1 mutant could not be detected in the liver only after extremely high doses of 5×10^6 pfu were applied, then it was found at day 2 post infection (p.i.) with no liver damage as confirmed by the serum alanine aminotransferase (ALT) level. Following this unprecedented *in vivo* attenuation strategy of knocking out a major pathogenicity determinant, we postulated that, including this strategy in coronavirus-based vaccine vectors will tremendously improve the safety profile.

3.4.3.2 NS2a gene

MHV ORF2 encodes a polypeptide of about 30 kd molecular weight with no potential N-glycosylation site and lacking all the credentials of a membrane protein (Luytjes et al., 1988;

Shieh et al., 1989). The gene product of ORF2 now designated as ns2 (Cavanagh et al., 1990) was detected by *in vitro* translation of mRNA2 (Leibowitz et al., 1982; Siddell, 1983). A protein of same size was seen in MHV infected cells but its identity was yet established (Siddell et al., 1981). An ns2-specific polyvalent antiserum confirmed the identity of ns2-gene product and showed its localization to the cytosol (Bredenbeek et al., 1990; Zoltick et al., 1990) of MHV infected cells. As with most non-structural proteins, the actual function of the ns2 gene product is not known but was proposed to have a role in replication, possibly as an RNA binding protein (Luytjes et al., 1988). This proposal was contrasted when it was demonstrated that the ns2-gene products were not essential for virus replication in transformed murine cells (Schwarz et al., 1990). Hence, we reasoned that deleting the ns2 gene will first and foremost not affect replication of the vector in cell culture and will add to the attenuation of the vector *in vivo* thereby improving the safety while making life difficult for the vector in an eventual coinfection with wild type virus. Furthermore, this deletion will increase the vector cloning capacity by providing space.

3.4.3.3 Hemagglutinin-Esterase (HE)

The presence of this glycoprotein on the virion surface of coronaviruses appear to be optional since it is present in Bovine coronavirus (BCV), Turkey coronavirus (TCV), Human coronavirus (HCV) and some strains of MHV but absent in Infectious bronchitis virus (IBV) and the Transmissible gastroenteritis virus (TGEV) (Deregt et al., 1987; Garwes and Reynolds, 1981; Hogue and Brian, 1986; King et al., 1985; Stern and Sefton, 1982). Coronavirus HE proteins are sialic acid-binding type I envelope glycoproteins showing sialate-*O*-actylesterase activity (Smits et al., 2005). For BCV, both HE and the spike recognise the same receptor determinant of 9-O-acetyl-neuraminic acid on host cells (Popova and Zhang, 2002). The HE protein is expressed by a minority of MHV strains such as the MHV-S, some isolates of JHM (Yokomori et al., 1991) while the tissue adapted MHV-A59 genome encodes an HE which because of multiple mutations does not get expressed at the protein level and the gene is referred to as a pseudogene (Shieh et al., 1989). Additionally, expression of the viral HE is not necessary for virulence in vivo as the MHV-A59 still causes hepatitis, encephalitis as well as demyelination though it does not express HE. The fact that HE expression is nonessential for the viral life cycle, indicate that this protein may have a role during infection of the host (Smits et al., 2005). There have been speculations that HE may be important in acute and or chronic disease induced by MHV, possibly by influencing the cellular tropism (Yokomori et al., 1995; Yokomori et al., 1993) or may help

spread the virus by increasing attachment or exit from the cell (Kienzle et al., 1990). Additionally, an increased mortality and increased infection observed in neurons was associated with a JHM variant that expressed high levels of HE as against a variant that expressed less HE (Yokomori et al., 1995). The pathogeneses of isogenic recombinant viruses expressing either a wild-type HE, or HE with the acetyl esterase activity knocked out or no HE expression at all, were compared. Interestingly both viruses that expressed HE polypeptides with or without a functional acetyl esterase activity were more virulent when inoculated intracranially into mice (Kazi et al., 2005). This outcome would support a model in which HE may enhance virus attachment and spread through sialic acid lectin receptors (Kienzle et al., 1990) and could be suggestive that the sialic acid binding domain is separate from the acetyl esterase domain. However, since the MHV HE displays 30% homology to the HA1 subunit of the hemagglutinin-esterase fusion protein of influenza C virus (Luytjes et al., 1988) and because a recent report show that the specificity of the neuraminidase (esterase) for a particular type of sialic acid determines the cell subtype infected within the respiratory tract and hence the pathogenic outcome (Matrosovich et al., 2004), the HE may play a significant role in coronavirus biology. Additionally, the optional nature of this protein suggest that it is dispensable and may serve an unknown luxury function that could be costly (Lissenberg et al., 2005). Furthermore, since the MHV-A59 HE is not essential for replication in tissue culture, we reasoned that its eventual deletion will improve vector safety as well as provide additional space for heterologous antigen cloning.

3.4.3.4 ORFs 4 and 5a

Group 2 coronaviruses largely represented by MHV possess four group specific genes, two (genes 4 and 5a) of which are located between the 3’ end of the spike (S) gene and the small envelope (E) gene and the other two (NS2a and HE) located between the 3’ end of the replicase 1b and the 5’end of S gene. The functions of these genes in MHV biology have not been clearly defined. In an elegant report using RNA targeted recombination, it was clearly demonstrated that the deletion of these genes from the MHV genome did not affect the viability in cell culture but was attenuating in the natural host (de Haan et al., 2002a).

In view of the stringent scrutiny for safety of candidate vaccine vectors and in order not to compromise our vaccine vectors, we out rightly further deleted the remaining two group specific genes namely genes 4 and 5a from the vector genome. This action further improves the

safety profile of MHV-based vectors and increases the cloning capacity for heterologous gene insertion.

3.4.3.5 The small envelope gene (E)

Coronavirus E protein is an integral membrane protein and a minor component in the virus particle with a size range of 76 – 109 amino acids (Godet et al., 1992; Liu and Inglis, 1991; Yu et al., 1994). Infectious bronchitis virus (IBV) E was reported to localize at the Golgi when expressed on its own or when co-expressed with IBV M protein in IBV infected cells (Corse and Machamer, 2000) and the C-terminal of this protein is implicated to target the Golgi (Corse and Machamer, 2002). Furthermore, whereas the SARS E protein has been shown to localize at the Golgi at least during early infection (Liao et al., 2006), MHV E was found to locate at the ER-Golgi intermediate compartment (Raamsman et al., 2000).

Biochemical characterization indicates that coronavirus E protein may undergo post translational modifications. In deed all E proteins have conserved cysteine residues located on the carboxy side of the long hydrophobic domain implicating a functional importance. For example the IBV and SARS E are both palmitoylated on one or more cysteine residues (Corse and Machamer, 2002; Liao et al., 2006). Until recently, the status of MHV E post translational modification was not defined. Almost simultaneously, two groups reported observations that MHV-A59 E protein lacking all three cysteines exhibited an increased rate of degradation compared to the wild-type protein, suggesting that palmitoylation is crucial for the stability of the protein and for it to function as a vesicle morphogenetic protein in order for the assembly subunit to assume configurations that can mobilize into secretory lipid vesicle and virion (Boscarino et al., 2008; Lopez et al., 2008).

Coronavirus E protein is crucial for virion assembly. Expression of coronavirus E and M proteins together led to the formation of VLPs which were morphologically indistinguishable from the coronavirions (Baudoux et al., 1998; Bos et al., 1996; Corse and Machamer, 2000; Lim and Liu, 2001; Vennema et al., 1996). Furthermore, expression of E protein on its own was shown to release E containing membrane vesicles (Maeda et al., 1999). Though the exact function of E protein in virion assembly is yet established, its low abundance in virion and VLPs might imply a function in membrane curvature. More recently the transmembrane domain of IBV E was shown to be required for efficient release of viral particles (Machamer and Youn, 2006).

The E proteins of MHV and SARS have been implicated to induce apoptosis (Yang et al., 2005). More so the involvement of E protein in virion morphogenesis was demonstrated using targeted RNA recombination in which mutations were introduced at the C terminal hydrophilic tail of MHV-E protein. This led to the production of thermolabile mutants that appeared aberrant and heterogeneous in morphology (Fischer et al., 1998). Furthermore, a mutant MHV with deletion of the E protein showed tiny plaques with low growth rate and titers (Kuo and Masters, 2003) demonstrating the importance of E protein for efficient propagation of a ΔE-coronavirus.

In summary, due to the pivotal role impacted by MHV E protein in VLPs formation, it was necessary to delete this gene from the newly generated MHV-based vaccine vectors in order to increase safety by producing particles that are replication competent but propagation deficient. The deletion of the E gene also allowed additional cloning space for heterologous antigen or immunostimulatory cytokine insertion. We have therefore constructed coronavirus-based vectors with the MHV-A59 background carrying the following deletions replicase encoded nsp1Δ99-ΔNS2a-ΔHE-Δgene4-Δgene5a and ΔE. We are confident to crown these vectors as highly biosafe vaccine vectors because in case of any eventual attempt to revert into wild-type, we anticipate at least five rounds of recombination and in addition, these vectors will have to compensate the 99 nucleotides deletion in the MHV-nsp1 coding region.

4 Aims and scientific questions addressed in this project

The major aim of this project was to develop recombinant murine coronavirus-based vectors expressing different combinations of model antigens and immunostimulatory cytokines. This was to be achieved through cloning of well-characterized antigens and immunostimulatory molecules which can be properly evaluated due to the availability of established experimental protocols. The selection of a particular combination of antigens and cytokines was intended to provide efficient insights into some pertinent questions pertaining to the evaluation of any promising vaccine candidate vector.

Second, in order to package the vector RNA into virus-like particles (VLPs) that could be employed for immunization studies, a stable packaging system had to be developed.

Thirdly, we aimed to assess the immunogenicity of MHV-based vectors in well-defined animal models: The LCMV gp33 epitope system has been chosen to assess both antiviral and antitumour responses in mice. Furthermore, the human Melan-A epitope has been selected to test relevant antitumour immune responses in humanized HLA-transgenic mice.

5 Original article

5.1 Generation of Recombinant Coronaviruses Using Vaccinia Virus as the Cloning Vector and Stable Cell Lines Containing Coronaviral Replicon RNAs

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Book chapter - pages 237-254

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My contribution to this work included the performance of experiments, validation and writing of protocols indicated at sections 3.1.3 and 3.3.2 of this chapter.

Generation of Recombinant Coronaviruses Using Vaccinia Virus as the Cloning Vector and Stable Cell Lines Containing Coronaviral Replicon RNAs

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Abstract

Coronavirus reverse genetic systems have become valuable tools for studying the molecular biology of coronavirus infections. They have been applied to the generation of recombinant coronaviruses, selectable replicon RNAs, and coronavirus-based vectors for heterologous gene expression. Here we provide a collection of protocols for the generation, cloning, and modification of full-length coronavirus cDNA using vaccinia virus as a cloning vector. Based on cloned coronaviral cDNA, we describe the generation of recombinant coronaviruses and stable cell lines containing coronaviral replicon RNAs. Initially, the vaccinia virus-based reverse genetic system was established for the generation of recombinant human coronavirus 229E. However, it is also applicable to the generation of other coronaviruses, such as the avian infectious bronchitis virus, mouse hepatitis virus, and SARS coronavirus.

Key words: Coronavirus; RNA virus; reverse genetics; vaccinia virus; full-length cDNA; *in vitro* transcription; recombinant coronaviruses

1. Introduction

The extraordinarily large size of the positive-stranded coronavirus RNA genome posed a significant obstacle for the establishment of coronavirus reverse genetic systems based on cloned full-length cDNA. Conventional cloning techniques using plasmid DNA cloning vectors were not suitable to stably accommodate large coronaviral cDNAs. Moreover, in numerous cases, specific coronaviral cDNA sequences turned out to be resistant to cloning in conventional plasmid DNAs or

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were unstable upon propagation in prokaryotic hosts. Finally, however, several laboratories succeeded in establishing coronavirus reverse genetic systems based on full-length cDNA ***(1–3)***. Not surprisingly, those researchers had to solve the problem of "cDNA instability" and, accordingly, the solutions they provided are all based on nonconventional approaches.

The reverse genetic system described here is based on the use of a vaccinia virus as a cloning vector that replicates in eukaryotic cells. Vaccinia virus is a large DNA virus with a genome size of approximately 200 kbp that is able to stably accommodate foreign DNA sequences of coronavirus genome size (27–31 kb). The basic techniques required to clone (Section 3.1), modify (Section 3.2), and rescue (Section 3.3) recombinant coronaviruses are described. One application of the system, namely, the generation of coronavirus replicon RNAs and cell lines, is described in Section 3.4.

2. Materials

1. QiaexII Gel elution kit (Qiagen).
2. Buffer A (10 mM Tris-Cl pH 9.0, 1 mM EDTA).
3. MagNA Lyser Instrument, MagNa Lyser Green Beads (Roche).
4. Phosphate-buffered saline (PBS).
5. 0.25% (w/v) trypsin.
6. 36% sucrose.
7. Sorvall or Beckman ultracentrifuge, AH-629 or SW-28 rotors.
8. RNase-free DNase.
9. Proteinase K, PCR grade (Roche).
10. Proteinase K digestion buffer (1X concentration: 100 mM Tris-Cl pH 7.5, 5 mM EDTA, 0.2% (w/v) SDS, 200 mM NaCl).
11. RNase-free water.
12. T4 DNA Ligase (high-concentrate; Fermentas).
13. Pulse field gel instrument and equipment.
14. Lipofectin, Lipofectamine2000 (Invitrogen).
15. Sonication water bath (Branson 3210).
16. RiboMax large-scale RNA production system—T7 (Promega).
17. $m^7G(5')ppp(5')G$ cap analog (30 mM).
18. LiCl solution (7.5 M LiCl, 50 mM EDTA pH 7.5).
19. Sodium dodecylsulfate (SDS).
20. Electroporation instrument (e.g., BioRad Gene Pulser, 0.4-cm electroporation cuvettes.
21. Cell culture medium chemicals for GPT^+ selection: (a) mycophenolic acid (MPA), 10 mg/ml in 0.1 M NaOH (400X stock); (b) xanthine, 10 mg/ml in 0.1 M NaoH (40X stock) and (c) hypoxanthine, 10 mg/ml in 0.1 M NaOH (667X stock).
22. Cell culture medium chemicals for GPT^- selection: 6-thioguanine (6-TG), 1 mg/ml (1000X stock).

23. Cells: BHK-21, CV-1, D980R ***(4)***.
24. Viruses: Vaccinia virus vNotI/tk ***(5)***, fowlpox virus.

3. Methods

3.1. Cloning of Coronavirus cDNA in Vaccinia Virus

This section describes the steps involved in the cloning of a full-length coronavirus cDNA. Starting from viral RNA, a set of plasmid DNAs should be generated together covering the full-length coronavirus genomic sequence. The plasmid insert cDNAs are then assembled by *in vitro* ligation to obtain a full-length coronavirus cDNA fragment. This fragment will be inserted into a vaccinia virus genome, again by *in vitro* ligation. The cloned full-length coronavirus cDNA in vaccinia virus is then amenable to mutagenesis by vaccinia virus-mediated homologous recombination.

3.1.1. Generation of Plasmid DNAs Covering a Coronavirus Full-Length cDNA

1. Analyze the coronavirus genome for useful naturally encoded endonuclease restriction sites that can later be used to ligate cloned cDNA inserts. Preferably use restriction enzymes that produce nonpalindromic sticky ends with at least three nucleotide (nt) overhangs. Avoid the use of restriction enzymes that generate blunt ends, since ligation efficiencies of blunt end fragments are low. If there are no useful sites available at particular regions of the cDNA sequence, restriction sites may be generated that introduce silent nucleotide changes. Alternatively, introduce sites at the border of the coronavirus cDNA fragments for restriction enzymes that cleave outside of their recognition sequence and orientate the sites so that cleavage occurs in the coronavirus cDNA region **(Fig. 1A)**.
2. Generate a set of plasmid DNAs covering the entire coronavirus cDNA using standard plasmid DNA cloning techniques. cDNA insert fragments should have a size of approximately 5 kbp. Make sure that the cDNA fragment borders are flanked by appropriate endonuclease restriction sites in order to release the cloned cDNA fragments by endonuclease digestion (see above). If particular plasmid clones appear unstable upon propagation in *Escherichia coli*, change the plasmid backbone, preferably to a low copy plasmid. If plasmid DNAs remain unstable upon propagation proceed with the cloning of the remaining part of the coronavirus cDNA and insert the respective unstable cDNA sequence on the vaccinia virus level by vaccinia virus-mediated recombination using RT-PCR cDNA fragments (see Section 3.2).
3. The cDNA fragments corresponding to the 5'- and 3'-end of the genome should contain additional sequences as follows **(Fig. 2)**. To facilitate cloning into the vaccinia virus genome by *in vitro* ligation with *Not*I-cleaved vaccinia virus DNA (see Section 3.1.4) both end fragments should contain an *Eag*I or *Bsp*120I site.

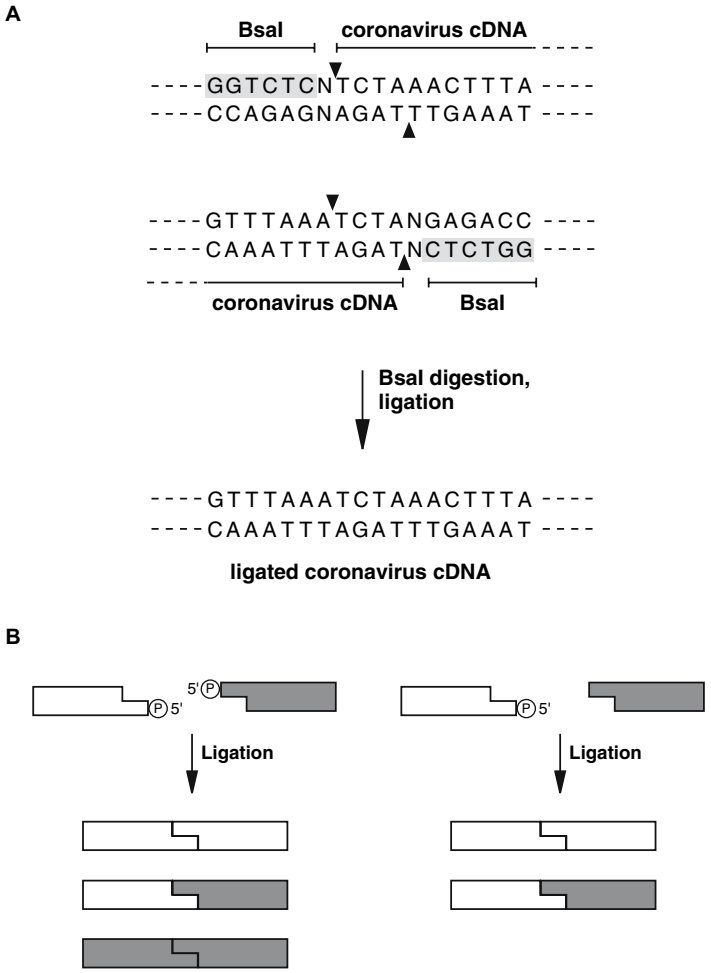

Fig. 1. Strategies to minimize the number of possible ligation products: (A) Ligation of two coronavirus cDNA fragments using *Bsa*I restriction endonuclease. *Bsa*I recognition sequences can be engineered adjacent to the coronavirus cDNA to obtain *Bsa*I-cleaved cDNA ends without heterologous sequences. The sticky ends are not palindromic and are comprised of a coronavirus-encoded sequence. The subsequent ligation reaction is directional and gives rise to only one possible reaction product. (B) The use of alkaline phosphatase to reduce the number of possible ligation products is illustrated. The left panel shows a conventional ligation using cDNA fragments with palindromic sticky ends. In this case three different ligation products are possible. The right panel shows a ligation reaction if one cDNA fragment has been dephosphorylated with alkaline phosphatase prior to the ligation reaction. In this case only two ligation products are possible.

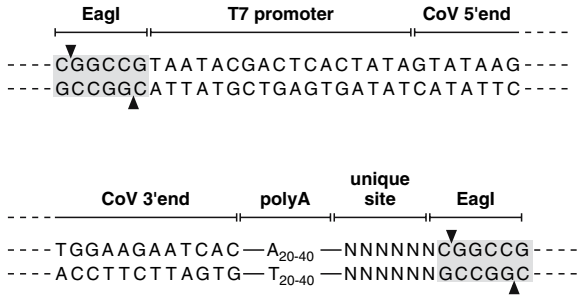

Fig. 2. Structure of 5'- and 3'-ends of cloned full-length coronavirus cDNA. Upstream of the coronavirus 5'-end there should be an *Eag*I or *Bsp*120I restriction endonuclease site to allow insertion of the cDNA into the *Not*I site of the vaccinia virus genomic DNA by *in vitro* ligation. Between the *Eag*I or *Bsp*120I site and the 5'-end of the coronavirus cDNA sequence there should be a bacteriophage T7 RNA polymerase promoter and one G nucleotide (if not yet present at the 5'-end of the coronavirus genome) for the initiation of the *in vitro* transcription reaction. Downstream of the 3'-end of the coronavirus genome, a stretch of 20–40 A nucleotides (synthetic poly(A) tail) and a unique (i.e., not present in the coronavirus genomic sequence) restriction endonuclease site should be cloned. The unique restriction site is needed for the generation of runoff *in vitro* transcripts. Furthermore, an *Eag*I or *Bsp*120I site is needed to insert the cDNA into the *Not*I site of the vaccinia virus genome by *in vitro* ligation. If the coronavirus sequence does not encode an *Eag*I or *Bsp*120I site, the unique site is not needed since *Eag*I or *Bsp*120I cleavage can be done to produce templates for the generation of runoff *in vitro* transcripts.

(DNA cleaved with *Eag*I or *Bsp*120I can be ligated with *Not*I cleaved DNA.) Furthermore, the 5'-end fragment should contain a promoter for the bacteriophage T7 RNA polymerase and one G nucleotide (if not yet present at the genomic 5'-end of the coronavirus genomic RNA) for the proper initiation of the *in vitro* transcription. The 3'-end fragment should encode a stretch of A nucleotides (approximately 20 nt) followed by a restriction site that is not present in the coronavirus cDNA sequence.

3.1.2. Assembly of Long cDNA Fragments by in Vitro Ligation

1. Design a strategy for the sequential assembly of cloned cDNA inserts. Examples have been described for the construction of full-length human coronavirus 229E (HCoV-229E), avian infectious bronchitis virus (IBV), and mouse hepatitis virus, strain A59 (MHV-A59) cDNAs ***(3,6,7)***.
2. Liberate cloned insert cDNA fragments from plasmid DNAs by restriction endonuclease cleavage. Start with 50–100 μg plasmid DNA. Isolate the cDNA fragments by gel purification using standard agarose gels. Avoid exposure of cDNA fragments to UV light during the isolation process (*see* **Note 1**). If palindromic sticky

ends are present at the cDNA fragment termini, the number of possible ligation products can be reduced by dephosporylation of one ligation partner by alkaline phosphatase treatment **(Fig.** 1**B)**. Note that the ends of cDNA fragments corresponding to the 5'- and 3'-genomic end should be cleaved with *Eag*I or *Bsp*120I and dephosphorylated by alkaline phosphatase treatment.

3. Ligate cDNA fragments in analytical scale using high-concentrate T4 DNA ligase. Leave ligation reaction overnight at room temperature. Analyze ligation products on standard agarose gels. The samples should be heated to 65°C for 5 min before loading on the gel.
4. If the ligation reaction worked efficiently in the analytical scale, use the same conditions for a preparative scale ligation. Analyze an aliquot of the reaction on a standard agarose gel. If inefficient ligation is encountered repeatedly, revise the assembly strategy by using alternative restriction sites.
5. If the preparative ligation reaction was efficient, purify the desired ligation fragments by gel purification (avoid UV exposure; *see* **Note 1**) using the QiaexII gel elution procedure (Qiagen).
6. Use the ligated and purified cDNA fragments from step 5 for further ligation reactions with further cDNA fragments until a full-length cDNA fragment has been obtained. Alternatively assemble fragments to obtain a set of not more than two or three cDNA fragments together encompassing the entire coronavirus cDNA sequence (*see* **Note 2**).

3.1.3. Preparation of Vaccinia Virus DNA

This section describes the preparation of purified vaccinia virus DNA that can be used: (i) for the *in vitro* ligation with the assembled coronavirus full-length cDNA (see Section 3.1.4), and (ii) as template for *in vitro* transcription reactions (see Section 3.3.1). The protocol describes the vaccinia virus purification and subsequent DNA preparation in a preparative scale (virus derived from 10 to 20 150-cm^2 flasks of infected BHK-21 cells). However, the protocol can also be down-scaled.

1. Grow 10 to 20 150-cm^2 flasks of BHK-21 cells to 80% confluency and infect with vaccinia virus. Vaccinia virus infection should be done with an appropriate multiplicity of infection (MOI) to obtain complete a cytopathic effect (CPE) 3 days postinfection (p.i.).
2. Three days p.i. freeze cells by putting the flasks into a freezer for at least 2 h. Thaw, collect, and pellet cells (1000 rpm, 5 min, 4°C). Wash cell pellet with PBS. Resuspend concentrated infected cells in 1 ml Buffer A per 150-cm^2 tissue culture flask.
3. Fill MagNA Lyser green bead tubes to maximum filling level of 1 ml.
4. Homogenize using MagNA Lyser machine speed 5000 1 × 20 sec (*see* **Note 3**).
5. Centrifuge at 1000 rpm 4°C for 2 min.

6. Pipette supernatant into a clean fresh tube. Treat supernatant with 0.1 volume 0.25% (w/v) trypsin and incubate at 37°C for 20 min.
7. Adjust the trypsin-treated cell homogenate with buffer A to a volume of 18 ml and carefully overlay an 18-ml 36% (w/v) sucrose cushion in a 36-ml ultracentrifugation tube and centrifuge (13,500 rpm at 4°C for 80 min; Sorvall or Beckman ultracentrifuge, Rotor AH 629 or SW 28).
8. Discard the supernatant and resuspend the pellet in 0.4 ml Buffer A.
9. Digest with RNase-free DNase (1–5 U) for 20 min (*see* **Note 4**) and then stop DNase treatment by adjusting the solution to 10 mM EDTA and incubate for 10 min at 65°C.
10. Add 1 vol of 2X Proteinase K digestion buffer (final concentration is 1X proteinase K digestion buffer) and 4 μl proteinase K; incubate at 50°C for 2 h.
11. Extract DNA with 1 vol phenol/chloroform/isoamylalcohol (25:24:1), mix gently (do not vortex! *See* **Note 5**), and centrifuge (14,000 rpm, 5 min, room temperature, Eppendorf centrifuge). Take the water phase and perform a second round of DNA extraction with 1 vol chloroform/isoamylalcohol (24:1), mix gently (do not vortex!) and centrifuge (14,000 rpm, 5 min, room temperature, Eppendorf centrifuge).
12. Take the water phase and add 2.5 vol 100% ethanol, mix gently (do not vortex!), and pellet the DNA by centrifugation (14,000 rpm, 5 min, room temperature, Eppendorf centrifuge) (*see* **Note 5**).
13. Discard the supernatant and wash the DNA with 70% ethanol; centrifuge again (14,000 rpm, 5 min, room temperature, Eppendorf centrifuge).
14. Discard the supernatant completely and resolve the DNA in 50–200 μl RNase-free water.

3.1.4. Ligation of Insert cDNA with Vaccinia Virus DNA

This section describes the integration of the assembled coronaviral cDNA fragments (Section 3.1.2) into the vaccinia virus genome by *in vitro* ligation. Inserted DNA fragments can be a full-length coronavirus cDNA or two or three cDNA fragments that can be ligated just prior to the ligation to the vaccinia virus genomic DNA. As parental virus we recommend the vaccinia virus v*Not*I/tk that encodes a unique *Not*I cloning site ***(5)***.

1. Set up a standard *in vitro* ligation reaction if the insert DNA consists of more than one fragment. This procedure is only recommended if the number of possible ligation products is limited (e.g., by avoiding palindromic sticky ends or by limiting the number of possible ligation products by using one dephosporylated DNA end per ligation reaction). The reaction should include a standard ligation buffer (including ATP) and can have a volume of up to 100 μl. Let the reaction go for 1–2 h at room temperature while preparing the *Not*I-cleaved vaccinia virus DNA (step 2). We recommend a molar ratio of insert:vaccinia virus DNA of 1:1 and a prior test of the reaction on an analytical scale.

2. Cleave vaccinia virus v*Not*I/tk DNA with *Not*I for 1–2 h at 37°C. The volume of the reaction can be up to 50 μl.
3. Mix the ligation reaction and the *Not*I restriction reaction and adjust buffers to 1X concentrations (1X ligation buffer and 1X *Not*I restriction buffer). Add fresh T4 ligase and *Not*I enzymes and incubate overnight at room temperature (*see* **Note 6**).
4. Heat the reaction to 65°C for 5 min, centrifuge (14,000 rpm, room temperature, Eppendorf centrifuge). Take the supernatant, add fresh *Not*I enzyme, and incubate at 37°C for 1–2 h (see **Note 7**).
5. Analyze the reaction products (or an aliquot thereof) on a pulse field gel. Prior to loading the sample(s) heat to 65°C for 5 min to achieve appropriate separation of DNA fragments in the pulse field gel.
6. Store ligation products at –20°C.

3.1.5. Rescue of Recombinant Vaccinia Viruses Containing Full-Length Coronavirus cDNA Insert

1. Seed CV-1 cells in a six-well dish 1 day before transfection. Cells should be 80% confluent for optimal transfection efficiency.
2. Infect 80% confluent CV-1 cells with fowlpox virus (MOI 1–10) for 1–2 h (*see* **Note 8**).
3. Transfect ligation reaction from Section 3.1.4 (without any further purification) into fowlpox virus-infected CV-1 cells using Lipofectin as described by the manufacturer (Invitrogen). Do not vortex at any time and use cut pipette tips when handling vaccinia virus DNA (*see* **Note 5**).
4. After 3–4 h trypsinize cells and seed them together with fresh (uninfected) CV-1 cells (4:1 excess of fresh CV-1 cells) into a 96-well plate.
5. At 5–10 days posttransfection collect cells and medium from wells displaying CPE (this is the first vaccinia virus stock) (*see* **Note 9**).
6. Transfer half of the first vaccinia virus stock to fresh CV-1 cells plated in a six-well dish. Wait until full CPE becomes apparent and collect the second vaccinia virus stock.
7. To analyze the obtained recombinant vaccinia viruses take half of the second vaccinia virus stock, pellet cells, and prepare DNA from the cell pellet according to Section 3.1.3, steps 10–14 (use 1X Proteinase K buffer).
8. To confirm the identity of recombinant vaccinia viruses perform Southern blot, PCR, and/or sequencing analyses.

3.2. Modification of Coronavirus cDNA by Vaccinia Virus-Mediated Homologous Recombination

The cloned coronavirus cDNA is amenable to mutagenesis by vaccinia virus-mediated homologous recombination. Two steps of homologous recombination

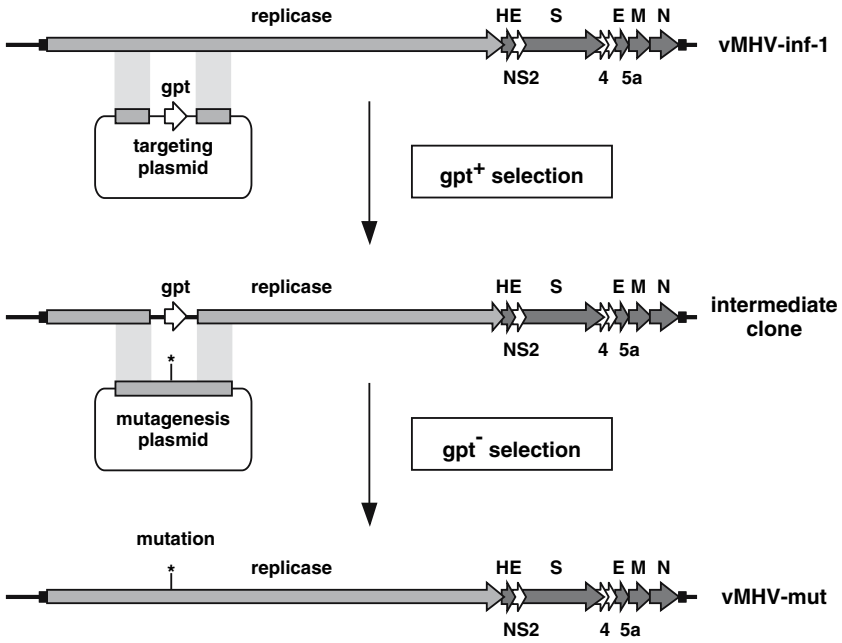

Fig. 3. Modification of the coronavirus cDNA. The modification of the cloned coronavirus cDNA by vaccinia virus-mediated homologous recombination is illustrated. The parental vaccinia virus vMHV-inf-1 is used in combination with a targeting plasmid to target the region of interest in the clone cDNA. Upon gpt^+ selection an intermediate clone is obtained that is subjected to a second round of recombination with a mutagenesis plasmid encoding the mutation of choice. The final mutant cDNA clone can be obtained after gpt^- selection. Note that the intermediate clone can also be used to introduce other mutations in the targeted region by using a different mutagenesis plasmid.

are performed using appropriate plasmid DNAs (**Fig. 3**). The first "targeting" plasmid DNA contains a sequence of approximately 500 bp corresponding to a region encoded upstream of the region that should get targeted for mutagenesis (left flank), the *E. coli* guanosin-phosphoribosyltransferase (gpt) gene located downstream of a vaccinia virus promoter and a sequence of approximately 500 bp corresponding to a region encoded downstream of the region that should be targeted for mutagenesis (right flank).

The targeting plasmid is used to target the region of interest for a second round of recombination using a "mutagenesis" plasmid that contains the left and right flank of the targeting plasmid and, between the flanks, the region of interest encoding the desired mutation. Both plasmids can be constructed by standard cloning techniques. We routinely use the plasmid pGPT-1 ***(4)*** that

is based on pBluescriptKS$^+$ (Stratagene) and contains a fragment encoding the *E. coli* gpt gene downstream of a vaccinia virus promoter cloned into the pBluescriptKS$^+$ multiple cloning site. The plasmid pGPT-1 is available from the authors upon request. Note that vaccinia virus-mediated homologous recombination also works when a linear DNA fragment, such as an RT-PCR product, is used instead of a plasmid DNA ***(7)***.

3.2.1. Vaccinia Virus-Mediated Homologous Recombination

This section describes the procedure of vaccinia virus-mediated homologous recombination. The protocol is used to generate a "transfection" stock containing recombined viruses at a ratio of approximately 1:1000 (recombinant viruses:parental viruses). The transfection stock is subsequently subjected to plaque purification under gpt$^+$ or gpt$^-$ selection (see Sections 3.2.2. and 3.2.3) to obtain stocks of recombinant vaccinia virus clones.

1. Seed CV-1 cells in a six-well dish so that they are 80–95% confluent on the day of infection.
2. Infect with parental vaccinia virus at an MOI of 1. Incubate for 1–2 h at 37°C.
3. At 1–2 h postinfection transfect 5 μg of the targeting or mutagenesis plasmid using Lipofectin or Lipofectamine 2000 according to the manufacturer's (Invitrogen) instructions.
4. Wash cells at 3–6 h posttransfection and culture cells for 2–3 days until full CPE becomes apparent.
5. Prepare a virus stock (which is the "transfection stock") from the infected/transfected culture by scraping the cells off the plate in 0.5 ml of the culture medium. Store at –20°C.

3.2.2. Targeting the Region of Interest: Selection of Recombinant gpt$^+$ Vaccinia Viruses

1. Seed CV-1 cells in a six-well dish so that they are 80–95% confluent on the day of infection.
2. Replace culture medium with gpt$^+$ selection medium (MEM containing 5% FCS, antibiotics and 25 μg/ml MPA, 250 μg/ml xanthine, and 15 μg/ml hypoxanthine) at least 6 h prior to infection.
3. Freeze-thaw the vaccinia virus transfection stock (see Section 3.2.1.) three times using dry ice on ethanol and sonicate for 1–5 min immediately prior to infection (*see* **Note 10**).
4. Infect CV-1 cells with different dilutions (10^{-2}, $10^{—3}$, and 10^{24}) of the transfection stock (*see* **Note 11**).
5. Culture cells for 2–3 days in the gpt$^+$ selection medium. Pick plaques as soon as they are easily detectable (usually on day 2 p.i.) by marking them at the bottom of

the dish, followed by scraping the cells of the plaque off the dish, and aspirate in 100 µl of medium using a standard pipette (*see* **Note 12**).
6. Perform another two rounds of gpt^+ plaque selection (steps 4 and 5) by infecting the CV-1 cells with 5–20 µl of a picked plaque. Always perform freeze-thaw cycles and sonication prior to infection.
7. After the third round of plaque selection infect CV-1 cells in a six-well dish with half of a picked plaque and culture cells until full CPE. Store half of this stock for further use and use the other half for DNA preparation and analysis of the recombinant vaccinia virus clone (i.e., PCR, Southern blot, sequencing).

3.2.3. Inserting the Mutation of Choice: gpt Negative Selection

1. Seed D980R cells in a six-well dish so that they are 60–80% confluent on the day of infection (*see* **Note 13**). Cells can be seeded in gpt^- selection medium containing 0.5 µg/ml 6-TG. The cells should be cultured in gpt^- selection for at least 6 h prior to infection.
2. Perform plaque selection as described in Section 3.2.2. steps 4–7. The only differences are the cells (D980R cells) and the gpt^- selection medium.

3.3. Rescue of Recombinant Coronaviruses from Cloned Full-Length cDNA

The rescue of recombinant coronaviruses is based on two steps. First, a full-length coronavirus RNA is produced using the genomic DNA of a vaccinia virus containing the full-length coronavirus cDNA insert as a template for *in vitro* transcription. Second, the recombinant full-length RNA is transfected into eukaryotic cells. Within these cells the coronavirus replication cycle will be initiated by translation of replicase gene products from the transfected RNA and, finally, recombinant coronaviruses are released into the tissue culture supernatant.

3.3.1. Generation of Infectious Full-Length Coronavirus RNA by in Vitro Transcription

1. Prepare vaccinia virus DNA from purified virus stocks as described in Section 3.1.3.
2. Cleave the vaccinia virus DNA (1–10 µg) with the restriction enzyme for which a unique recognition site downstream of the synthetic poly(A) tail has been introduced (**Fig. 2**).
3. Extract DNA with 1 vol phenol/chloroform/isoamylalcohol (25:24:1), mix gently, and centrifuge (14,000 rpm, 5 min, room temperature, Eppendorf centrifuge). Take the water phase and perform a second round of DNA extraction with 1 vol

chloroform/isoamylalcohol (24:1), mix gently, and centrifuge (14,000 rpm, 5 min, room temperature, Eppendorf centrifuge).
4. Take water phase and precipitate the cleaved vaccinia virus DNA by adding 1/20 vol of 5 M NaCl and 2.5 vol of 100% ethanol and centrifuge (14,000 rpm, 5 min, Eppendorf centrifuge). Do not overdry vaccinia virus DNA.
5. Wash DNA pellet with 70% ethanol; centrifuge again.
6. Completely remove the supernatant and resolve the DNA in 10–20 μl RNase-free water.
7. Set up the *in vitro* transcription reaction using the RiboMax Kit (Promega) as follows (*see* **Note 14**):

5X transcription buffer	10 μl
m^7G(5')ppp(5')G cap analog (30 mM)	5 μl
GTP (100 mM)	0.7 μl
ATP, CTP, UTP (100 mM), each	3.75 μl
Template DNA (1–10 μg)	*x* μl
RNase-free water	*y* μl
Enzyme mix (RNasin, T7 RNA pol.)	5 μl
Total	50 μl

7. Incubate at 30°C for 2 h.
8. Add 2 ml of RNase-free DNase, incubate at 37 °C for 20 min. Either store the reaction at –80 °C until transfection or (optional) precipitate the RNA (steps 9–11).
9. Add half the volume of LiCl solution and freeze the sample for at least 30 min.
10. Pellet RNA by centrifugation (14,000, 15 min, 4 °C, Eppendorf centrifuge)
11. Wash RNA pellet (should appear yellowish) with 70% ethanol and resolve in RNase-free water. Store at –80 °C.
12. Analyze the RNA on an agarose gel containing 1% SDS. Stain the RNA after gel electrophoresis with ethidium bromide.

3.3.2. Rescue of Recombinant Coronaviruses

1. One day before RNA transfection seed BHK-21 (*see* **Note 15**) cells so that there are 5×10^6 to 1×10^7 BHK-21 cells for each transfection. RNA transfection will be performed by electroporation.
2. Trypsinize, collect, and pellet 5×10^6 to 1×10^7 BHK-21 cells (centrifuge 1000 rpm, 5 min, 4 °C). Perform all further steps on ice.
3. Wash cells with 20 ml ice-cold PBS. Make sure that cells are well separated. Take one drop to count the cells and pellet the rest again (1000 rpm, 5 min, 4 °C).
4. Resolve 5×10^6 to 1×10^7 BHK-21 cells in 0.8 ml ice-cold PBS and fill into a 0.4-cm electroporation cuvette.

5. Add RNA and electroporate with two pulses (settings on BioRad Gene Pulser: Resistance = ∞, 230 V, high-capacity 1000 μF) (*see* **Note 15**).
6. Transfer the electroporated cells from into a 10-cm culture dish with 10 ml warm culture medium and add 1×10^6 fresh cells that are susceptible for the coronavirus that should be rescued (e.g., murine 17Cl1 cells for MHV rescue, human MRC-5 cells for the rescue of HCoV-229E).
7. Change the medium after 3–6 h when cells have attached to the bottom of the culture dish.
8. Recombinant coronaviruses should be released into the tissue culture medium between days 1 and 3 postelectroporation. Check for released virus on days 1–3 by transferring part of the supernatant onto susceptible fresh cells. Store culture supernatant for further analysis at –80 °C.

3.4. Coronavirus Replicon RNAs

Replicon RNAs are autonomously replicating RNAs encoding: (i) all replicative proteins required for the expression of a functional replication complex, and (ii) *cis*-acting elements required for the recognition of the replicon RNA by the replicase complex. Usually replicon RNAs are devoid of sequences leading to production of progeny particles. Coronavirus replicon RNAs differ from those of other positive-stranded RNA viruses in that they have to encode the nucleocapsid protein, which has been shown to be important for efficient coronavirus RNA replication ***(8***,***9)***.

It has been shown for HCoV-229E and SARS-CoV replicons that stable cell lines can be generated if the replicon RNA mediates the expression of a selection marker ***(4***,***10)***. Two selection markers, conferring neomycin/G418 ***(4)*** or blasticidin ***(10)*** resistance, have been used successfully for establishing stable coronavirus replicon cell lines. The HCoV-229E replicon RNA encodes the neomycin resistance gene inserted downstream of the nonstructural protein (Nsp) 1 and a sequence encoding a "2A-like" autoprocessing peptide. The 2A-like autoprocessing peptide mediates a co-translational liberation of a slightly modified Nsp1 carboxyterminus and subsequent translation of the neomycin resistance gene. In order to ensure translation of the remaining Nsps of the replicase gene (Nsps 2–16), an internal ribosomal entry site (IRES) derived from the encephalomyocarditis virus (EMCV) has been placed upstream of the Nsp2-coding sequence.

The SARS-CoV replicon RNA contains a gene encoding a fusion protein comprising the green fluorescent protein (GFP) and the blasticidin deaminase (GFP-BlaR) that has been cloned downstream of the replicase gene as a separate transcription unit under the control of the transcription regulatory sequence (TRS) of the SARS-CoV spike gene. In both cases, transfection of *in vitro*

synthesized replicon RNA into eukaryotic cells and subsequent selection using G418 or blasticidin resulted in the establishment of stable cell lines containing actively replicating coronavirus replicon RNAs. To facilitate the detection of replicon-containing cell lines, green fluorescence resulting from replicon-mediated GFP expression has been used. For the SARS-CoV replicon RNA this has been achieved by the use of the GFP-BlaR fusion protein ***(10)***. To achieve GFP expression by the HCoV-229E replicon RNA, the GFP gene has been inserted as a separate transcription unit downstream from the replicase gene, driven by the TRS of the HCoV-229E spike gene ***(4)***.

Coronavirus replicon cell lines can be used as a noninfectious system to analyze coronavirus replication and transcription or to identify and evaluate replicase inhibitors. The following protocols describe the generation of coronavirus replicon cell lines and their use in the evaluation of coronavirus replicase inhibitors.

3.4.1. Generation of Coronavirus Replicon Cell Lines

1. Based on a full-length coronavirus cDNA cloned in vaccinia virus, a replicon RNA-encoding cDNA can be generated using vaccinia virus-mediated homologous recombination as described in Section 3.2.
2. Generate replicon RNA by *in vitro* transcription as described in Section 3.3.1.
3. Introduce the replicon RNA into a host cell line of choice (*see* **Note 16**) by electroporation as described in Section 3.3.2 (steps 1–5).
4. Plate the transfected cells in normal growth medium. Change the medium after 3–6 h when cells have attached to the bottom of the culture dish and continue to culture the cells for 1–2 days in growth medium without selection pressure. Split if necessary.
5. Start the selection of stable lines at antibiotic concentrations only slightly above the level at which nontransfected cells die (*see* **Note 17**).
6. Increase the antibiotic concentration gradually during the following 2–3 weeks until resistant colonies appear.
7. Pick colonies for subculture in separate wells and test them for maintenance of replicon RNA. Expression of a reporter protein, such as GFP, by the replicon RNA facilitates the screening of replicon RNA-containing resistant colonies.
8. When stable clones have been obtained, further culturing can be done under low selection pressure (*see* **note 18**). Replicon cells can be stored in liquid nitrogen.

3.4.2. Identification and Evaluation of Coronavirus Replicase Inhibitors Using Replicon Cell Lines

1. Seed the replicon cells in selection medium so that they are 50–70% confluent on the next day. You can use 96- , 24- , or 6-well dishes.
2. Prior to adding antiviral compounds, wash the cells and culture them in standard medium without selection drugs.

3. Add graded doses of antiviral compound(s) to the cells and culture them for 1–3 days (*see* **Note 19**). For comparison include nontreated cells and culture them under identical conditions.
4. In order to assay for cytotoxicity of candidate inhibitors and to determine the selectivity index, include a cytotoxicity/cell viability test. This can be done with replicon cells or the respective parental cell line.
5. Determine GFP expression on days 1, 2, and 3 posttreatment by fluorescence microscopy and flow cytometry (*see* **Note 20**).

4. Notes

1. We found that DNA that has been exposed to UV light is difficult and sometimes impossible to clone into vaccinia virus DNA. When purifying DNA fragments from agarose gels, cut small slices at the edges of the fragment band out of the gel and stain them with ethidium bromide. Use UV light to visualize the borders of the DNA band in the slices and mark the position. Insert the slices back into the gel and cut the piece of agarose between the two marked positions out of the gel. The DNA recovered from those agarose pieces have not been exposed to UV light and are easily clonable in vaccinia virus.
2. It is possible to insert more than one DNA fragment into the vaccina virus genome by *in vitro* ligation ***(37)***. Up to three DNA fragments can be ligated prior to adding *Not*I-cleaved vaccinia virus DNA (see Section 3.1.4, step 1). However, we recommend this procedure only if the fragments can be efficiently ligated (a full-length cDNA fragment should be visible in agarose gels). Furthermore, the number of possible ligation products should be minimized using strategies illustrated in **Fig. 1**.
3. In order to prepare vaccinia virus, DNA the virus particles have to be liberated from cells and cell debris. This can be achieved by using a tight Dounce homogenisator or, as described here, by using the MagNA Lyser protocol. To establish appropriate conditions we recommend doing a pilot experiment in which several conditions are compared. After homogenization check for virus titers and decide for the most vigorous homogenization conditions that still leave the virus particles intact.
4. This step results in DNase digestion of free DNA (mostly of cellular origin) and will leave the DNA in virus particles intact.
5. Vaccinia virus genomic DNA has a size of approximately 200 kbp. Standard pipette tips are usually too narrow and pipetting will result in shearing the DNA. To avoid this, cut the pipette tips to generate an opening of about 2–3 mm and avoid vigorous pipetting. Avoid drying the DNA. If the large vaccinia virus DNA is overdried it will no longer be possible to dissolve it in water.
6. The ligation of insert DNA fragment(s) and the *Not*I-cleaved vaccinia virus arms is facilitated by adding *Not*I into the ligation reaction ***(11)***. Religated vaccinia virus arms are recleaved by the *Not*I enzyme allowing a new round of ligation. The 5'- and 3'-ends of the insert fragment are cleaved with *Eag*I or *Bsp*120I

and dephosphorylated. Therefore: (i) insert-insert ligation is not possible (owing to dephophorylated ends), and (ii) *Eag* or *Bsp*120I-*Not*I ligation products (i.e., insert-vector ligation) are not recleavable with *Not*I. As a result the ligation-restriction reaction will drive the overall reaction toward an accumulation of insert-vector ligation products.

7. The ligation-restriction reaction may still contain a small proportion of unrecleaved vector-vector ligation products. These products may lead to functional vaccinia virus genomes without the insert cDNA fragment and may cause a high background in the rescue of recombinant vaccinia viruses (Section 3.1.5). We therefore recommend an additional *Not*I cleavage reaction after the ligation-restriction reaction.
8. Since the vaccinia virus genomic DNA is not infectious, a helper virus has to be provided to rescue recombinant vaccinia viruses from DNA. We recommend fowlpox virus as a helper virus, since a fowlpox virus infection is abortive in mammalian cells, but can still serve to rescue vaccinia virus from DNA. Therefore, the recovered viruses will be vaccinia virus only (and no fowlpox virus).
9. On days 2–4 p.i. the cells may look heavily infected, most likely owing to fowlpox virus infection. However, recombinant vaccinia virus cannot yet be expected in the cell culture. Just continue to cultivate the cells; most cell layers will recover. Usually, the first vaccinia virus-mediated CPE can be expected on day 5 p.i. and a peak is observed around day 7 p.i.. If cells get too confluent the medium can be changed.
10. Freeze-thawing and sonication is needed to release and separate vaccinia virus particles from the cells and cell debris. For selection of pure recombinant vaccinia virus this procedure is critical.
11. A ratio of 1:1000 of recombinant vaccinia viruses:parental vaccinia viruses can be expected. Thus, under selection pressure, single plaques should appear on CV-1 cell layers that have been infected with a 10^{-3} dilution of the transfection stock. At 2 h p.i., an overlay of 1% low-melting agarose in selection medium can be made. This is done to reduce the risk of contamination of recombinant plaques with parental virus. Because most vaccinia virus is contained within an infected cell within the time frame (2–3 days) of the selection, agarose overlays are usually not necessary.
12. Six plaques is a reasonable number to pick. There is a limited risk of picking "false" plaques or plaques contaminated by parental virus that necessitates selection of a few plaques in parallel.
13. D980R cells grow fast and vaccinia virus plaques are not as easily recognizable as on CV-1 cells. During the selection it is necessary to keep cells in good condition to facilitate the formation of easily detectable plaques. The risk of overgrowth can be reduced by seeding cells at a lower density, and cell death from starvation can be reduced by replacing the medium with fresh selection medium on day 2 p.i..

14. It is possible, although not recommended by the manufacturer, to use the Promega RiboMax Kit to generate capped *in vitro* transcripts. One simply has to add a cap structure analog to the reaction. In the given *in vitro* transcription protocol the ratio of $m^7G(5')ppp(5')G$ cap analog to GTP is 2:1.
15. The optimal conditions for the electoporation of long RNA molecules are dependent on the cells and the electroporation device. BHK-21 cells are known to be suitable for efficient RNA electroporation and should be the first choice. We recommend doing a pilot optimization to determine optimal conditions for RNA transfection. It is now well established that the coronavirus nucleocapsid (N) protein facilitates the rescue of recombinant coronaviruses in several systems ***(2,6,7)***. We recommend co-electroporating an mRNA encoding the coronavirus N protein (5–10 μg N mRNA produced by *in vitro* transcription). It is even more efficient to generate and use a BHK-21-derived cell line stably expressing the N protein ***(7)***.
16. We observed replicon RNA replication in a wide variety of eukaryotic host cells. Although coronaviruses are usually species specific, coronavirus replicon RNAs are able to replicate in many cell lines once introduced into the host cell cytoplasm by transfection. Cell lines tested in our laboratory (using the HCoV-229E replicon) include cells of human (e.g., MRC-5, HeLa cells) and animal (e.g., BHK-21, 17clone1, L929 cells) origin.
17. We recommend determining the lowest concentration of the selection drug where nontransfected cells die for the cell line of choice.
18. Replicon cell lines based on commonly used cells such as baby hamster kidney (BHK) or Chinese hamster ovary (CHO) cells are generally easy to culture. To increase the number of cells expressing a high level of replicon-derived transcripts it is important to split the lines often enough to maintain them constantly subconfluent. GFP is a convenient marker to determine the percentage of GFP-expressing cells by flow cytometry.
19. Depending on the cell density and the stability of the compound it might be necessary to change the medium daily.
20. GFP is a valuable reporter protein to determine the percentage of green fluorescent cells as a marker for the percentage of cells with actively replicating RNA or to determine the mean fluorescence as a value that indicates GFP expression levels. Some inhibitors may lead to a reduced overall number of green fluorescent cells, whereas some inhibitors may just reduce the mean fluorescence. To generate more quantitative data on the inhibitory effect of a compound and to gain some insight into the kinetics of inhibition, other reporter proteins, such as luciferase proteins or alkaline phosphatase, may be used.

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5.2 Towards a coronavirus-based HIV multigene vaccine

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Towards a coronavirus-based HIV multigene vaccine

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Abstract
Human immunodeficiency virus (HIV) infection represents one of the major health threats in the developing world. The costly treatment of infected individuals with multiple highly efficient anti-HIV drugs is only affordable in industrialized countries. Thus, an efficient vaccination strategy is required to prevent the further spread of the infection. The molecular biology of coronaviruses and particular features of the human coronavirus 229E (HCoV 229E) indicate that HCoV 229E-based vaccine vectors can become a new class of highly efficient vaccines. First, the receptor of HCoV 229E, human aminopeptidase N (hAPN or CD13) is expressed mainly on human dendritic cells (DCs) and macrophages indicating that targeting of HCoV 229E-based vectors to professional antigen presenting cells can be achieved by receptor-mediated transduction. Second, HCoV 229E structural genes can be replaced by multiple transcriptional units encoding various antigens. These virus-like particles (VLPs) containing HCoV 229E-based vector RNA have the ability to transduce human DCs and to mediate heterologous gene expression in these cells. Finally, coronavirus infections are associated with mainly respiratory and enteric diseases, and natural transmission of coronaviruses occurs via mucosal surfaces. In humans, HCoV 229E causes common cold by infecting the upper respiratory tract. HCoV 229E infections are mainly encountered in children and re-infection occurs frequently in adults. It is thus most likely that pre-existing immunity against HCoV 229E will not significantly impact on the vaccination efficiency if HCoV 229E-based vectors are used in humans.

Keywords: *AIDS, vaccination, coronavirus, HIV*

Abbreviations: *HIV, human immunodeficiency virus; DCs, dendritic cells; AIDS, acquired immunodeficiency syndrome; HCoV, human coronavirus; MHV, mouse hepatitis virus*

Introduction

Prophylactic vaccines against several viral infections have been developed over the last centuries leading to the eradication of smallpox and protecting many people from diseases such as measles, rubella, mumps and polio. However, a number of diseases remain against which current vaccines are suboptimal or unavailable. Furthermore, there is growing need to develop therapeutic vaccines which may boost specific immune response to persistent viruses such as human immunodeficiency virus (HIV). The critical first step in the development of antiviral vaccines is the identification of the dominant antigens contributing to the different stages of the infection, i.e. initial replication at the site of entry, spread in the host and establishment of a persistent infection. The methodology for the identification of antigens and the characterization of immunodominant epitopes is well-established and has been further advanced by approaches from the fields of proteomics and genomics (Chakravarti et al. 2000). However, the major bottle-neck in the development of new and effective vaccines is the delivery of antigens to cellular components of the immune system that initiate protective antiviral immunity. The unmatched capacity of dendritic cells (DCs) to sample antigen at sites of pathogen entry, transport pathogens and their immunogenic components to secondary

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lymphoid organs and to initiate activation of T cells make them the ideal target cell for antimicrobial vaccines (Steinman and Pope 2002). The excellent capacity of DCs to prime antiviral T cell responses can be readily shown *in vitro*, i.e. few DCs can activate large numbers of virus-specific T cells in a mixed lymphocyte culture (Macatonia et al. 1989; Nonacs et al. 1992). An important prerequisite for the initiation of immune responses *in vivo* is the translocation of antigens from peripheral sites into secondary lymphoid organs (Zinkernagel et al. 1997). The high potency of DCs to induce protective antiviral immunity against the non-cytopathic lymphocytic choriomeningitis virus (LCMV) *in vivo* has been shown in studies where only 100–1000 DCs presenting a specific viral antigen have to reach secondary lymphoid organs for the induction of protective antiviral T cell responses (Ludewig et al. 1998; Ludewig et al. 2000b). DC-induced CTL responses develop rapidly and DC-immunized mice are protected against acute systemic and peripheral viral challenge (Ludewig et al. 1999). Likewise, adoptive transfer of DCs pulsed with inactivated HIV-1 into severely immunocompromised mice reconstituted with human PBL resulted in the induction of protective anti-HIV-1 responses (Lapenta et al. 2003). Moreover, vaccination of SIV-infected rhesus monkeys with a cellular DC vaccine significantly suppressed viral replication (Lu et al. 2003). A recent study in untreated HIV-1 infected individuals revealed that DC-based vaccination can elicit potent immune responses against immunodeficiency viruses in humans (Lu et al. 2004).

The efficacy of vaccines can be enhanced if optimal activation/maturation of DCs is achieved. For example, DC maturation via toll-like receptor ligands augments the activation of cytomegalovirus- and HIV-specific T cell responses *in vitro* (Lore et al. 2003). Likewise, the efficiency of various vaccine formats can be greatly enhanced if activation of DCs *in vivo* is mediated via co-delivery of immunostimulatory oligonucleotides (Sparwasser et al. 1998; Ludewig et al. 2000a) or binding to heat shock proteins (Cho et al. 2000). Incorporation of DC-activating chemokines or factors prolonging DC survival into genetic vaccines has been shown to enhance the immune response against recombinant rabies virus (Pinto et al. 2003) or HIV gp120 (Biragyn et al. 2002). A potent and effective HIV vaccine should thus directly deliver antigens to DCs and induce their activation/maturation.

HIV infection and immunity

Prevention of HIV infection. The thorough knowledge of the biology of HIV that has been generated over the last two decades has paved the way for a rational vaccine design. Furthermore, the progress in the understanding of the basic immunological mechanisms underlying antigen presentation (Steinman and Pope 2002), lymphocyte trafficking and activation (Luther and Cyster 2001), and immunological memory (Kaech et al. 2002) has been instrumental for the identification of the relevant parameters that ensure the induction of protective antiviral immunity. Accordingly, an efficient HIV vaccine should induce long-lasting, broad humoral and cellular responses against the immunodominant HIV antigens. In particular, the vaccine should (i) target and activate DCs, (ii) contain the immunodominant antigens recognized by CTL and Th cells, (iii) be able to display antigenic determinants that induce broadly neutralizing antibody responses, and (iv) be applicable via mucosal surfaces.

HIV-specific CTL and Th cell responses. CTL responses crucially contribute to control of immunodeficiency virus infection. Broad virus-specific CTL responses can be found in peripheral blood of HIV-infected humans (Betts et al. 2001; Addo et al. 2003) and the decline of plasma viral RNA during primary HIV infection is associated with the appearance of HIV-specific CTL (Borrow et al. 1994; Koup et al. 1994). Furthermore, transient *in vivo* depletion of CD8 T cells lead to a massive increase in viral load in SIV-infected monkeys, whereas extension of the depletion for more than 28 days elicited a progressive AIDS-like syndrome (Jin et al. 1999; Schmitz et al. 1999). HIV-specific Th cells can be detected in infected individuals (Pitcher et al. 1999). It is, however, not yet clear whether these cells extert direct antiviral effects. However, the good correlation of functional CD4 T cell responses against HIV (Rosenberg et al. 1997) or SIV (McKay et al. 2003) with the clinical status strongly supports the notion that intact Th cell responses are instrumental for long-term virus control. This is most likely mediated indirectly by stimulation of virus-specific CTL. Since most patients develop T cell responses against the HIV proteins env, gag or nef (Betts et al. 2001; Addo et al. 2003), a broadly applicable vaccine should elicit immune responses (at least) against these three immmunodominant antigens.

Broadly neutralizing antibodies. Non-neutralizing antibodies directed against viral proteins appear early after HIV infection, whereas neutralizing antibodies appear usually rather late after primary infection (Pilgrim et al. 1997). Furthermore, sera from HIV-infected individuals usually display only weak neutralizing activity against primary isolates (Moore et al. 1995). The fact that depletion of B cells in Rhesus monkeys significantly delayed the appearance of neutralizing antibodies but did not impact on the early viral clearance (Schmitz et al. 2003) supports the notion that neutralizing antibodies do not contribute

significantly during initial HIV infection. However, the presence of neutralizing antibodies may alter the clinical course of SHIV infection in macaques and prevents peripartal infection (Baba et al. 2000). Conventional vaccination approaches consistently failed to induce broadly neutralizing antibody responses (McMichael and Hanke 2003). Nevertheless, distinct monoclonal antibodies have been described that are capable of neutralizing a broad range of different HIV isolates, suggesting that such antibody responses might be induced once an adequate vaccination strategy has been developed (Moore et al. 2001). For example, altering the immunodominance pattern by using CD4-HIV envelope fusion constructs that expose normally occluded and conserved antigenic regions represents such an approach for the induction of broadly neutralizing antibodies (Fouts et al. 2003). An alternative strategy for the induction of antibodies that inhibit the infection of primary T cells with different primary HIV-1 isolates has been reported recently. This promising approach takes advantage of the highly conserved caveolin-1 binding domain of HIV-1 glycoprotein 41. Neutralization of the caveolin-1 binding site in gp41 efficiently blocks HIV-1 entry in a wide range of primary cells (Hovanessian et al. 2004).

Mucosal vaccination. HIV is predominantly transmitted via mucosal surfaces (Pope and Haase 2003). For example, SIV rapidly crosses the epithelial layers in the cervical mucosa and infects predominantly DCs and CD4 T cells (Spira et al. 1996). Following primary infection, the virus gains access to lymphoid organs and establishes persistent infection in CD4 T cells and macrophages. It appears that constant low-level exposure to virus (via mucosal surfaces?) is associated with resistance to HIV infection (Zhu et al. 2003). Mucosal vaccination may block transmission of intravaginally or intrarectally applied SIV (Amara et al. 2001; Belyakov et al. 2001; Veazey et al. 2003) indicating that an HIV vaccine should prevent the early stage of infection and elicit long-lasting mucosal immunity.

Coronavirus biology and suitability as viral vectors

Although immunogenic peptides or naked nucleic acid can elicit immune responses against HIV antigens, the use of viral vectors represents a superior strategy to deliver HIV antigens and/or immunostimulatory cytokines to specific target cells. However for several reasons, many virus vector systems are still limited in their ability to induce a broad and long-lasting antiviral immune response capable to prevent HIV infection and/or to reduce viral load. Moreover, the safety of DNA-based vectors such as adeno-associated-, retro- or lenti-viruses is a matter of concern, because they can integrate into the host cell genome (Dobbelstein 2003). Recombinant adenoviruses have been studied intensively as HIV vaccine candidates mainly because they can be produced to high titers. Nevertheless, high doses of recombinant adenovirus vectors have to be applied to induce antiviral immune response, most probably because they target antigens mainly to non-lymphoid organs such as the liver (Krebs et al. 2005). In contrast to viral vectors based on DNA viruses, the use of positive-stranded RNA virus-based vectors that replicate in the cytoplasm are considered as safe vectors because it is unlikely that sequences from these vectors can integrate into the host cell genome. Moreover, the safety is well documented for vectors based on widely used vaccine strains such as poliovirus (Crotty et al. 1999) or virus-like particles (VLPs) that contain replicon RNAs devoid of structural genes (Davis et al. 2000; Harvey et al. 2003). Although some of these vectors are able to target DCs and/or to induce mucosal immunity, their cloning capacity is generally restricted and the expression of multiple HIV antigens and/or immunostimulatory cytokines is limited.

Coronaviruses display a number of features that may be advantageous to overcome these limitations and, therefore, represent promising candidate vaccine vectors. Coronaviruses are enveloped viruses that are associated mainly with respiratory and enteric diseases. For example, human coronavirus 229E infects the mucosa of the upper respiratory tract and can cause common cold. Coronavirus genomes are the largest known autonomously replicating RNAs with a size of approximately 30 kb. About two thirds of the positive-stranded genome encode the replicase gene, which is comprised of two large open reading frames (ORFs). Upon infection, translation of the genomic RNA results in the synthesis of replicase gene-encoded polyproteins that are extensively processed by viral proteinases leading to the formation of a functional replicase–transcriptase complex within the cytoplasm of the infected cell (Ziebuhr et al. 2000). A hallmark of coronavirus genome expression is their unique transcription strategy. This strategy leads to the synthesis of multiple 3′ co-terminal subgenomic mRNAs, encoding mainly structural proteins. It has been shown that the synthesis of each subgenomic mRNA involves a discontinuous step by which the so-called 3′ body sequence is fused to the genomic 5′ leader sequence (Spaan et al. 1983). The fusion of leader and body sequences during discontinuous transcription is determined, at least in part, by *cis*-acting elements, termed transcription–regulatory sequences (TRS, also referred as transcription associated sequences). These elements are located at the 5′ end of the genome and at various 3′ proximal sites corresponding to the individual transcription

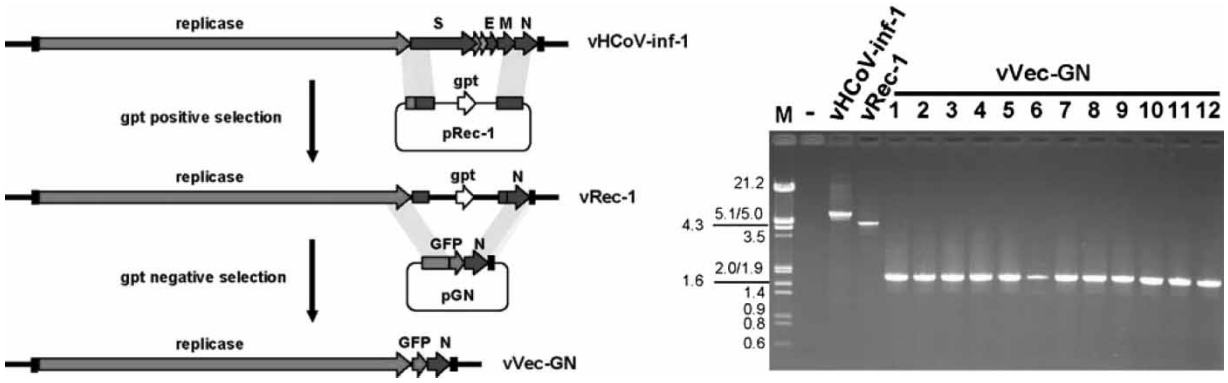

Figure 1. Mutagenesis of cloned coronavirus cDNA using vaccinia virus-mediated homologous recombination. (A) The generation of the recombinant vaccinia virus vVec-GN containing a HCoV 229E-based vector construct is illustrated. Two steps of recombination using the *E. coli* guanine phosphosribosyltransferase (gpt) as marker for positive and negative selection were performed. (B) The result of a PCR analysis from vaccinia viruses vHCoV-inf-1 (parental clone), vRec-1 (intermediate clone) and vVec-GN (desired final clone) is shown. PCR primers used in this analysis are located upstream and downstream of the region where recombination took place. Lanes 1–12 show 12 randomly picked recombinant vaccinia virus plaques obtained after gpt-negative selection, indicating the 100% recovery of desired genotypes. Notably, one vVec-GN clone was subjected to sequencing analysis of the entire coronavirus-based cDNA insert and no nucleotide changes were detected.

units. Although many studies have been performed to identify *cis*-acting sequences required for coronavirus transcription, exact borders of TRS elements have not yet been elucidated (Pasternak et al. 2001). However, short stretches of not more than 5–7 nucleotides within the TRS, called "core sequence", have been identified to determine the site of leader-body fusion of coronavirus subgenomic RNAs.

Because of the (molecular) biology of coronaviruses, coronavirus-based vectors are currently considered a promising system to genetically deliver multiple heterologous genes to specific target cells. First, coronaviruses are positive-stranded RNA viruses replicating in the cytoplasm without a DNA intermediary, making insertion of viral sequences into the host cell genome unlikely. Second, coronaviruses have the largest RNA genome known so far. Therefore, a cloning capacity of more than 6 kb is expected. Third, coronaviruses display a unique transcription process resulting in the synthesis of 6–8 subgenomic mRNAs, encoding mainly the structural genes. These genes, encoded at the 3′ third of the genome, can be replaced by multiple heterologous genes, e.g. immunogenic HIV antigens and/or immunomodulatory genes. Fourth, the receptors of human and murine coronaviruses (HCoV 229E and mouse hapatitis virus (MHV)) are expressed on human and murine DCs, respectively, indicating that efficient delivery (i.e. receptor-mediated uptake of VLPs) of heterologous genes to DCs can be achieved. Finally, the mucosal route is the natural way of coronavirus transmission.

Establishment of a reverse genetic system for coronaviruses

We have established a reverse genetic system for coronaviruses that allows the generation of recombinant coronaviruses (Thiel et al. 2001a, 2003; Coley et al. 2005). One of the main advantages of our system is that the cloned full-length coronavirus cDNAs are amenable to site-directed mutagenesis using vaccinia virus-mediated homologous recombination. This technique is well established and has been proven to represent an efficient and precise (on the nucleotide level) method to genetically modify recombinant coronavirus cDNAs. In Figure 1, we show one example to demonstrate the ease of using vaccinia virus-mediated recombination to genetically modify coronavirus cDNA inserts.

Generation of coronavirus-based multigene vector RNAs—transduction of human DCs. With the reverse genetic systems available, it is now possible to make use of the unique characteristics of coronavirus transcription to develop coronavirus expression vectors. The rationale of expressing heterologous genes using coronavirus-mediated transcription is to insert a transcriptional cassette, comprised of a coronavirus TRS located upstream of the gene of interest, into a coronavirus genome, minigenome or vector RNA. We have shown for human coronavirus vector RNAs that a region of at least 5.7 kb is dispensable for discontinuous transcription (Thiel et al. 2001b). This region contained all structural genes and, therefore, our vector RNAs are not infectious. We could demonstrate that it is possible to construct a human coronavirus vector RNA capable to mediate the expression of multiple heterologous proteins. Noteworthy, this vector RNA can be packaged to VLPs if the structural proteins are expressed in trans (Thiel et al. 2003). These results indicate that coronavirus-based vector systems might be useful for heterologous gene expression, especially for longer and multiple genes.

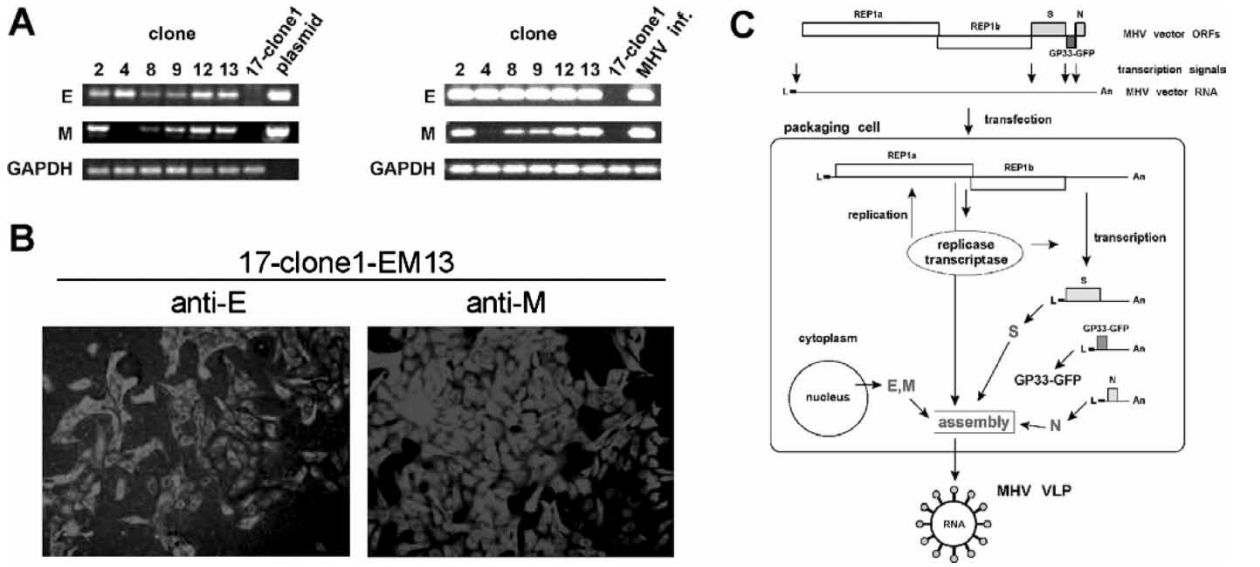

Figure 2. Packaging cell lines for the production of MHV VLPs. (A) Genomic DNA (left) or polyA-containing RNA (right) from cell clones (#2,4,8,9,12 and13) that were stably transfected with a plasmid DNA encoding the MHV *E* and *M* genes on separate transcription units were analyzed by *E* and *M* gene-specific PCR and RT–PCR, respectively. Control lanes are genomic DNA or polyA-containing RNA from parental murine 17-clone1 cells, plasmid DNA encoding E and M and polyA-containing RNA from MHV-infected 17-clone1 cells. (B) Immunofluorescence analysis of 17-clone1-EM13 cells using specific sera against MHV structural proteins E (left) and M (right). (C) Packaging strategy for the production of MHV VLPs using the MHV prototype vector MHV-Vec-A and the E- and M-expressing cell lines.

An important consideration for viral vaccine vectors is their potential for efficient delivery of their genetic material to specific target cells. For example, targeting of viral vaccine vectors to DCs is highly desirable in order to optimize vaccine efficacy. It is important to note that the HCoV 229E receptor, human aminopeptidase N (hAPN or CD13), is expressed at high levels on human DCs (Summers et al. 2001). This implies that HCoV 229E-based VLPs could be used to efficiently (receptor-mediated uptake) transduce these cells. We could demonstrate that HCoV 229E-based VLPs can be used to transduce immature and mature human DCs (Thiel et al. 2003). Therefore, this new class of safe, multigene vectors, based on HCoV 229E, represents a particularly promising tool to genetically deliver multiple antigens and immunostimulatory cytokines to human DCs.

A reverse genetic system for mouse hepatitis virus (MHV)—establishment of a murine model to assess the efficacy of coronavirus-based vaccine vectors

In order to study the efficacy of coronavirus-based vectors *in vivo*, a small animal model is desirable. Therefore, we first established a reverse genetic system for MHV. Again we made use of vaccinia virus as cloning vector to stably propagate the full-length cDNA of MHV (strain A59). Recombinant viruses obtained from this cDNA clone were indistinguishable from the parental MHV-A59 strain in tissue culture (growth kinetics, plaque size and RNA synthesis) and in MHV-related disease models in mice (Coley et al. 2005).

With the reverse genetic system for MHV it is now possible to generate MHV-based multigene vectors that resemble their HCoV-229E counterparts. Like all coronaviruses, MHV mediates the expression of multiple subgenomic mRNAs in the infected cell. Therefore, it is possible to use the coronavirus transcription mechanism for the generation of multigene MHV vectors. Furthermore, MHV is one of the best-studied coronaviruses *in vitro* and *in vivo*. MHV grows to high titers in tissue culture ($>10^9$ pfu/ml) and the requirements for the generation of VLPs are well understood. MHV also allows for the usage of a collection of well characterized inbred and transgenic mice and a variety of established immunological techniques, indispensable for the analysis of vector-induced immune responses. Finally, it has been shown that MHV-A59 can infect murine DCs (Turner et al. 2004) and therefore, recombinant MHV vectors in the context of a murine model can serve as a paradigm for the development and evaluation of coronavirus vaccine vectors.

An important prerequisite to study the efficacy of coronavirus vaccine vectors is the availability of VLPs that can be produced to high titers. Therefore, packaging cell lines must be established which mediate the expression of coronavirus structural proteins in trans. To this end, we have generated several cell clones derived from murine 17-clone1 cells, which stably express the MHV structural proteins E and M (EM-cells). These clones have been analysed for the expression of E and M by PCR using genomic DNA as template and RT–PCR using poly(A)-containing RNA as template (Figure 2A), and immunofluorescent

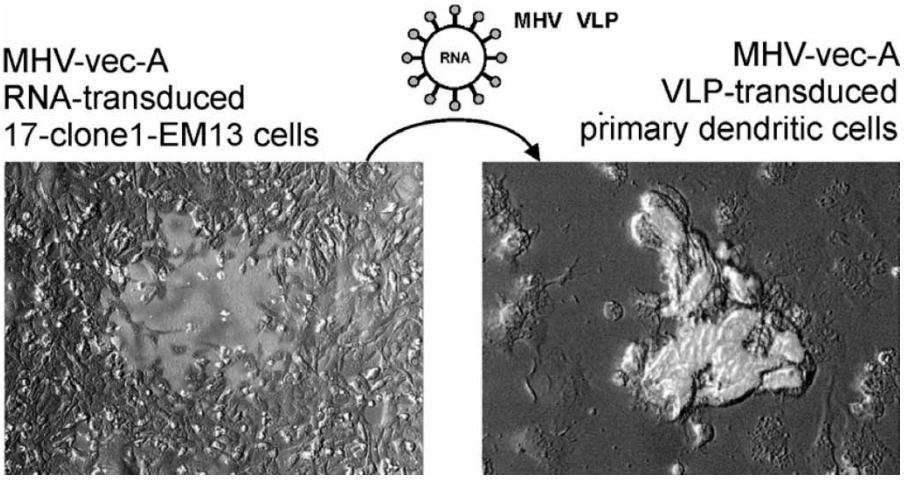

Figure 3. Generation of MHV VLPs and *in vitro* transduction of primary dendritic cells. MHV-Vec-A RNA was transfected into 17-clone1-EM13 cells by electroporation. Green fluorescence was detectable after 24 h (left). Supernatants from 17-clone1-EM13 cells were collected between 48 and 72 h following transfection and used to transduce primary DCs *in vitro*. Again, green fluorescence became apparent after 24 h (right) indicating that functional MHV VLPs have been produced in the 17-clone1-EM13 packaging cell line and that these VLPs can be used to transduce murine DCs.

microscopy (Figure 2B). Five out of six cell clones were found to contain and express both, E and M. Noteworthy, two considerations have been made before the construction of the EM cell line. First, the *E* and *M* genes in these cell lines are expressed by the cellular transcription of two separate mRNAs to minimize the possibility of reconstitution of infectious viruses by recombination of the MHV vector RNA with *E* and *M* gene mRNAs (Figure 2C). Second, in order to achieve high titer MHV VLP production, we decided to use a mouse cell line which is susceptible to MHV infection (17-clone1) for the stable transfection of *MHV E* and *M* genes. In this case, the packaging cells are susceptible to VLP-infection and we expect spread of MHV vector RNA throughout the tissue culture.

The E- and M-expressing cell lines can now be used to package MHV vector RNAs that encode (in addition to the replicase gene and the 5′ and 3′ *cis*-acting elements required for replication) the MHV structural proteins S and N (nucleocapsid protein). Therefore, we have generated a prototype MHV vector, designated MHV-Vec-A, containing the replicase gene, the 5′ and 3′ *cis*-acting elements required for replication, the structural protein S, the immunodominant CTL epitope GP33 of LCMV glycoprotein as a fusion protein with the green fluorescent protein (GP33-GFP) and the nucleocapsid protein. This vector RNA is currently being used to thoroughly assess the efficacy of VLP production in individual EM-packaging cells.

Our first experiments using MHV-Vec-A RNA for the transfection of the packaging cell line clone "17-clone1-EM13" showed that the transduction of these cells yields green fluorescent plaques indicating that our construct is functional, i.e. that the replicase complex, and the GP33-GFP fusion protein are produced. As expected, we could also observe syncytia in vector-transfected packaging cells, suggesting that a functional, cell fusion-mediating MHV S protein is present (Figure 3, left panel). Most importantly, the production of MHV VLPs is shown by the fact that transfer of supernatants from vector-transfected 17-clone1-EM13 cells to primary DC cultures leads to GFP expression in the target cells (Figure 3, right panel). Overall, these experiments provide proof-of-principle that the generation of MHV VLPs is feasible and that transgenes expressed by these replication-incompetent viruses can be targeted to DCs. We are currently in the process of testing the efficacies of MHV VLP production using the different 17-clone1-EM cell clones in order (i) to identify the best packaging cell clone; and (ii) to establish an optimized protocol for high titer VLP production.

Conclusions

The human immunodeficiency virus (HIV) pandemic with approximately 40 million people infected worldwide and more than 4 million deaths per year, represents a major human health problem. The majority of the infections occur in Africa and HIV-induced AIDS is the leading cause of death among adults aged 15–49 years in this region. Furthermore, the numbers of infections in developing countries such as India and China have been dramatically growing over the recent years. Antiviral drug treatment has increased life expectancy and quality in western countries, but this expensive medication is usually not accessible for infected individuals in developing countries. There is thus an urgent need for an efficient and affordable vaccine.

We believe that coronaviruses have tremendous capability as tools to deliver prophylactic and therapeutic proteins to disease-relevant target cells in human. In addition, this inherently safe vector system offers the opportunity to deliver multiple proteins in combination with immunostimulatory substances. The primary goal of the outlined approach is the establishment of the coronavirus vector system and its validation in a small animal model. If this approach is feasible and effective, we should commence with the development of HCoV 229E replicon-based VLPs encoding several HIV antigens (env, gag and nef) in combination with immunostimulatory molecules. The successfully established packaging strategy will be adapted to the HCoV 229E system and should allow production of recombinant HCoV 229E VLPs. Alternatively, pseudotyped MHV-based VLPs displaying a tropism for human DCs may be used for further studies. Safety and efficacy of this vaccine preparation should be tested in an adequate non-human primate model.

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6 Unpublished Data

6.1 Generation of MHV-based coronavirus vector particles

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Abstract

Coronavirus-based vectors are currently considered a promising means to genetically deliver multiple heterologous genes to specific target cells. Coronaviruses are positive-stranded RNA viruses replicating in the cytoplasm without a DNA intermediary, making insertion of viral derived sequences into the host cell genome unlikely. Coronaviruses have the largest known RNA genome therefore a cloning capacity of more than 6 kb is expected. They possess a unique transcription strategy resulting in the synthesis of 6-8 subgenomic mRNAs encoding structural and accessory genes, these genes can be replaced by multiple heterologous genes encoding antigens and cytokines. Finally, coronaviruses have the ability to infect professional antigen presenting cells such as dendritic cells (DCs) and macrophages. Here we report the construction and efficient propagation of murine coronavirus-based vectors that are devoid of all accessory genes, one structural gene (E) and have a deletion in the replicase-encoded non-structural protein 1(NspI) gene region. In order to produce virus like particles (VLPs) containing the vector RNA, a packaging cell line was made in which the gene encoding the deleted structural protein E was provided in trans. VLP production (10^6/ml) was successful, using the packaging cells. In vitro transduction experiment using these VLPs indicated efficient receptor mediated transduction of murine DCs.

Introduction

Vectored vaccines based on recombinant viruses or attenuated live virus vaccines represent the most effective immunogen mimicking real-life infection. Furthermore, recent concepts in immunology implicate a link between innate and adaptive immune responses arguing that the quality, quantity and longevity of adaptive immune responses is imprinted very early on after infection or vaccination (Pulendran and Ahmed, 2006). Therefore, for a successful vaccine approach, immunologically important cells such as DCs and macrophages that have the extraordinary ability in sensing pathogen-associated molecular patterns and orchestrating the upcoming immune responses involving both innate and adaptive arms of the immune system represent prime targets to deliver antigens and immunostimulatory molecules.

Coronaviruses are endowed with the intrinsic prowess of specifically targeting professional antigen presenting cells (pAPCs) capable of shaping the upcoming immune responses. Noteworthy, is the fact, that the receptors for most coronaviruses are expressed at high levels on pAPCs such as dendritic cells and macrophages. For example, the receptor for human coronavirus (HCoV) 229E, human amino peptidase N (CD13) and that of the mouse hepatitis virus (MHV), carcinoembyonic antigen cell adhesion molecule (ceacam) 1a, are both expressed on human and murine pAPCs respectively.

The biological properties of coronaviruses as depicted by the prototype MHV-A59 with a positive non-segmented RNA genome size of about 31kb which is infectious, capped and polyadenylated (Lai and Cavanagh, 1997) make this group of viruses particularly promising as vaccine vectors. The genes located downstream of the replicase gene encode mainly the structural and nonessential proteins, most of which can be deleted and replaced by heterologous antigens as reported for HCoV 229E (Thiel et al., 2003). Interestingly, MHV-A59 like other coronaviruses has its replication and transcription restricted to the cytosol of the infected cell without a DNA intermediary, therefore, highlighting the absence of any risk of integration of virus-derived nucleic acid sequences into the host cell genome. Furthermore, their transcription machinery mediates the production of a 3’ coterminal nested set of 6-8 subgenomic mRNAs (sgmRNAs). It has been shown that coronavirus sgmRNAs are transcribed from negative-strand RNA templates involving the fusion of non-contiguous sequences (Sawicki et al., 2007; Spaan et al., 1983). These mRNAs all contain a common leader sequence identical to the 5’ end of the genome and a body sequence composed of various lengths of the genomic 3’ end. This model of RNA synthesis termed discontinuous extension during subgenomic-length minus-strand

synthesis (Sawicki and Sawicki, 1998; Sawicki et al., 2007) is thought to involve small *cis*-acting elements known as transcription regulating sequences (TRS) that serve as transcription termination or pausing signals during negative-strand synthesis. Accordingly, the expression of heterologous genes by this transcription mechanism is achievable in the context of coronavirus genome, minigenome or replicon. This has been demonstrated in that the insertion of a transcriptional cassette composed of a gene of interest located downstream of a TRS element results in the synthesis of a corresponding subgenomic mRNA encoding the protein of interest (Alonso et al., 2002b; Curtis et al., 2002; Fischer et al., 1997; Thiel et al., 2001b; Thiel et al., 2003). Furthermore, though not fully understood, it is clear that different TRSs mediate different expression levels of mRNAs hence, coronavirus-based vectors could be used to simultaneously express differential amounts of multiple genes by simply cloning them downstream of various particular TRSs (Alonso et al., 2002a). Having the largest RNA genome known so far, coronaviruses can provide a capacity of 6-9 kb to clone multiple heterologous genes such as antigens and cytokines.

In a deliberate attempt to exploit basic immunological knowledge coupled to the natural biological characteristics of coronaviruses as promising vaccine candidates we report here the design and generation of multigene-RNA vaccine vectors based on MHV-A59. In the design of these vectors we have given priority to the safety of the vectors, considered the ability to manipulate the immune system in order to enhance immunogenicity and finally the possibility to produce higher titers for large scale immunization.

Material and Methods

Cells. CV-1, BHK-21 and L929 cells were purchased from the European collection of cell cultures (http://www.ecacc.org.uk/). CHO Tet-Off cells were purchased from Clontech, 17Clone1 (17Cl1) cells were kindly provided by S.G. Sawicki, Medical University of Ohio, Toledo, Ohio, USA, and D980R cells were a kind gift from G. L. Smith, Imperial College London, United Kingdom. Unless otherwise indicated all cells were maintained in minimal essential medium supplemented with 10% fetal calf serum (FCS) and 500u penicillin/ml, 100u streptomycin/ml (MEM-10).

Recombinant DNA and viruses. MHV-A59 was generated from a molecularly cloned cDNA (Coley et al., 2005) based on the Albany strain of MHV-A59. Coronaviruses and recombinant vaccinia viruses were propagated, titrated and purified as described (Coley et al., 2005; Hertzig et al., 2004; Thiel et al., 2001a). Mutant vaccinia viruses are based on the recombinant vaccinia virus vMHV-inf-1 (containing the full length MHV-A59 cDNA) and were generated using the reverse genetic system established in our laboratory as described previously (Coley et al., 2005; Eriksson et al., 2008). To perform vaccinia virus-mediated homologous recombination, the recombination plasmids were used in a first step to introduce the *Escherichia coli* guanine-phosphoribosyltransferase (GPT) gene into a genetically tailored region of the MHV cDNA backbone followed by a second recombination step using a plasmid with similar 5' and 3' homologous recombination sequences separated by a specific target gene of interest cloned downstream of a coronavirus TRS element.

Briefly vaccinia virus-mediated homologous recombination was done as follows. CV-I cells (5 $x10^5$) were infected (m.o.i=1) with vaccinia virus followed by transfection of 5µg plasmid DNA 1hr post infection using Lipofectin transfection reagent (Invitrogen) according to the manufacturer's recommendation. Cells were washed 4 hrs post transfection, overlaid with MEM-10 and incubated at 37°C for 2-3 days. When full cytopathic effect (cpe) became apparent the transfection stock was collected and recombinant vaccinia virus was selected by three rounds plaque purification under gpt positive or negative selection as appropriate. In order to isolate gpt positive vaccinia virus clones, gpt positive selection was done on CV-I cells in the presence of mycophenolic acid (25 µg ml^{-1}; Sigma), xanthine (250µg ml^{-1}; Calbiochem) and hypoxanthine (15 µg ml^{-1}; Calbiochem). Gpt negative selection was done on D980R cells in the presence of 6-

thioguanine (1µg ml^{-I}; Sigma) (Hertzig 2004; Eriksson 2008). The identities of all recombinant clones were confirmed by PCR and sequencing analysis.

To construct the recombinant vaccinia virus vMHV-GP-ΔEM encoding the vector MHV-GP-ΔEM the MHV-Inf-1 was modified by replacing the MHV nucleotides 21771-29623 by gpt using the plasmid pMHV-rec-1. This plasmid carries the *E. coli*-gpt gene flanked to its 5'end by a sequence corresponding to MHV nucleotides 21328-21771 and to the 3'end by a sequence corresponding to MHV nucleotides 29623-30115. The resulting gpt positive vaccinia virus clone vMHV-1b-gpt-N was used to generate the recombinant vaccinia virus containing the MHV GP-ΔEM cDNA by inserting the MHV spike, the GP-GFP (encoding a fusion protein comprised of the lymphocytic choriomeningitis virus (LCMV-WE) T cell epitope GP33-KAVYNFATC and the enhanced green fluorescent protein) and the MHV nucleocapsid in a second recombination with the plasmid pMHV-vec-2-kk. This plasmid contains 5'- MHV nucleotides 21328-21771 containing TRS2 –MHV 23930-27964 MHV-spike gene and containing TRS4 - GP-GFP containing TRS7 -MHV 29623-30115 - 3' end. The resulting gpt negative vaccinia virus was used to rescue the MHV vector MHV-GP-ΔEM.

In order to clone the MHV-GP vector the vaccinia virus clone vMHV-1b-gpt-N was again modified by inserting the MHV spike, the GP-GFP and the MHV-M and N genes in a second round of recombination with the plasmid pMHV-vec-10-ΔE. This plasmid was composed of 5'-MHV nucleotides 21328-21771 containing TRS2 –MHV 23930-27964 containing TRS4 - GP-GFP containing TRS6-MHV 28969-30114- 3'. The resulting vaccinia virus-vMHV-GP was used to rescue the MHV vector MHV-GP.

In order to insert the gene encoding the murine granulocyte colony stimulating factor (mGM-CSF) between the MHV replicase and spike genes, the recombinant vaccinia virus vMHV-GP was modified using the plasmid pMHV-rec-2-gpt composed of 5' MHV nucleotides 21328-21771containing TRS2- *E. coli*-gpt- MHV nucleotides 23890 – 24452. The resulting gpt positive clone vMHV-1b-gpt-S-GP was modified in a second round of recombination with the plasmid pMHV-rec-2-mGM-CSF composed of 5' MHV nucleotides 21328-21771 containing TRS2- mGM-CSF-MHV nucleotides 23890 – 24452 and a gpt negative selection was performed. The resulting gpt negative vaccinia virus vMHV-GMGP was used to rescue the vector MHV-GM/GP.

To establish a stable MHV-E-expressing cell line, the MHV-E gene was amplified by PCR and cloned into the multiple cloning site of the plasmid pTRE2hyg-vector (Clonetech) with the primer pair forward 5'-ACTGGGATCCACCATGTTTAATTTATTCCTTACAG-3' and

reverse 5'-ACTGGCGGCCGCTTAGATATCATCCACCTCTAATAG-3', using the BamHI and NotI sites (underlined).

Rescue of MHV-based vectors from cloned cDNA. Recombinant MHV-based vectors were rescued from cloned cDNA using purified vaccinia virus DNA as template for in vitro transcription of recombinant MHV-based vector genomes as described (Eriksson et al., 2006a; Eriksson et al., 2008; Thiel et al., 2001a). In order to rescue recombinant coronavirus-based vectors and package them into virus-like particles (VLPs), the full length vector RNA was transfected into packaging cells by electroporation or by lipofection using DMRIEC (Invitrogen) or using Mirus reagent (Mirusbio.com) according to the manufacturer's recommendation. The transfection was incubated in minimum essential medium supplemented with 5% Tet-approved FBS (Clontech), 500u penicillin/ml, 100u streptomycin/ml (Tet-induction MEM) at 37°C 5% CO_2 for about 2 days. MHV-based vector particles released into the supernatant within the next 24-48 hrs were collected and stored at -80°C until further usage. Titration of supernatant was performed on murine L929 cells as described (Zust et al., 2008).

Amplification of MHV-based vector particles in 150 cm^2 cell culture flasks. Fresh packaging cells were seeded in 150 cm^2 culture flasks with packaging cell line growth medium (MEM-10 containing 100µg/ml G418, 100µg/ml Hygromycin and 2µg/ml Doxycycline) a day before, so that the cells were 60% confluent the next day. The cells were transduced with MHV-vector particles and were incubated for at least 6hrs at 33°C. Growth medium was changed thereafter by adding 20ml fresh low pH DMEM medium (Dulbeco's modified eagle medium, supplemented with 0.75g/l $NaHCO_3$, 8.45g/l NaCl, 10ml 2M hepes pH6.6 (in dH_2O)/l, 5% Tet-approved FCS (Clontech), 5% tryptose phosphate broth, 500u penicillin/ml, 100u streptomycin/ml) and cells were further incubated at 33°C until full cpe became apparent (between 60 – 72hrs). MHV-vector stocks were obtained by collecting and freezing of transduced cells and cell culture supernatant, followed by thawing and centrifugation at 1500 rpm, 5 min. at 4°C (Sorval). The cell free VLP containing supernatant was stored -80°C until further usage.

Generation of high titer VLP stocks. 1.2 x 10^8 packaging cells trypsinized to make a single cell suspension were seeded in a roller bottle (850cm^2 Falcon, Becton Dickinson) in 75 ml packaging cell line growth medium. The bottle was rotated at 0.1 rotation circle per minute overnight so as to obtain the best distribution of the cells. The cells were transduced with VLP supernatant at a low m.o.i. (0.01-0.001) and incubated for 6 hrs at 33°C 5% CO_2 in minimal amount of medium just enough to cover the cells (20ml of VLP supernatant and fresh low pH DMEM). Thereafter the medium was changed by 30ml of fresh low pH DMEM. The cells were examined every day for signs of starvation and the rate of MHV-vector spreading. In case of signs of starvation, 2/3 volume of the growth medium was replaced by fresh low pH DMEM. MHV-vector stocks were prepared when full cpe became apparent (54 - 60hrs) as described above.

Polyethylene glycol VLP concentration. Polyethylene glycol 6000 (PEG-6000) was used to concentrate MHV-vector stocks. Briefly, 100 ml MHV-vector containing supernatant was centrifuged at 1500 rpm 4°C, 7 min. to clear the MHV-vector stock of remaining cell debris. The MHV-vector supernatant (100 ml) was introduced into a 250 ml Erlenmeyer conical flask and 7.4ml ice cold 5M NaCl was carefully added. Thereafter, 54 ml 30% (w/v) PEG-6000 in NTE (10mM Tris, pH6.5: 1mM EDTA; 100mM NaCl) was slowly added and the reaction was placed on a magnetic stirrer at 200 rpm for 30 min at 4°C. The mixture was spun (11200 rpm, Sorvall SS-34 rotor or 15000xg) for 30 min. The supernatant was carefully decanted and the pellet resuspended in the required volume of MEM supplemented with 2%FCS. The resuspended pellet was transferred into a sterile falcon tube and centrifuged at 1500 rpm for 5 min at 4°C. Aliquots of the supernatant were frozen at -80°C.

RT-PCR. RT-PCR analyses were performed in order to confirm the identity and stability of MHV-based vectors. To do this total RNA from MHV-vector-transduced cells was prepared using Trizol-reagent (Invitrogen) according to the manufacturer's recommendation. The RNA was used to synthesise cDNA using SuperScript™ II RT (Invitrogen) and oligo dT primer (sequence 5'TTTTTTTTTTTTTTTT-3') according to the manufacturer's recommendation. PCR amplification was performed and the products were analyzed by agarose gel separation and sequencing using standard protocols. The following primers were used to analyze the integrity of the GP-GFP gene and the mGM-CSF gene. GP-GFP: forward 5'-GTGATGAGTAG GAGGACACCAGG-3', reverse 5'-CTCGTGTAACCGAACTGTAGTATG-3'; mGM-CSF: forward 5'–TTGTTGGCGATAGCCTAGTAAATG-3', reverse 5'–CTGCACCTTCGCAAATA TGCCATC-3'. Vector cDNAs were analyzed for the presence of MHV E gene Using the E-

specific primers forward 5'-ACTGGGATCCACCATGTTTAATTTATTCCTTACAG-3' and reverse 5'-ACTGGCGGCCGCTTAGATATCATCCACCTCTAATAG-3'.

Analysis of heterologous gene expression. GFP expression in MHV-vector transduced cells was assessed by fluorescence microscopy or fluorescence activated cell sorting (FACS) analysis. GM-CSF expression was assessed by ELISA (mouse GM-CSF ready-set go Cat. No. 88-7334, ebioscience.com) using the supernatant of MHV-vector transduced cells according to the manufacturer's recommendation.

Generation of tetracycline inducible cell lines. Two mouse cell lines, 17Cl1 and L929 cells, were selected due to their susceptibility to MHV infection. The cells were plated in 6 wells so that they were 80% confluent on the day of transfection. Cells were transfected with the regulator plasmid pTet-off (Clontech) using lipofectin (Invitrogen) according to the manufacturer's protocol and incubated at 37°C. The cells were allowed to divide for 48 hrs before 400µg/ml of G418 in MEM-10 was applied as selection pressure. G418 resistant clones were expanded and subjected to primary screening by evaluating the luciferase activity following a transient transfection with the luciferase control plasmid (pTREhyg2-Luc) containing the tetracycline responsive element (TRE) under the minimal CMV promoter in the presence or absence of 10ng/ml Doxycycline. Luciferase activity was assayed using the luciferase assay system (Promega) as recommended by the manufacturer and measured using an automatic luminometer (Spectra Fluor Plus, Tecan).

Results

MHV-based vector design, cloning and packaging concept

In order to establish a robust high titer MHV-vector stock preparation, we have carefully designed and cloned a number of prototype MHV-based vectors. First the MHV-GP-ΔEM vector was designed such that (i) it be devoid of two structural genes in order to be propagation deficient (ii) to lack all accessory genes in order to attenuate the vector and (iii) to introduce a model antigen (GP-GFP) to assess vector-mediated heterologous gene expression (fig. 1).

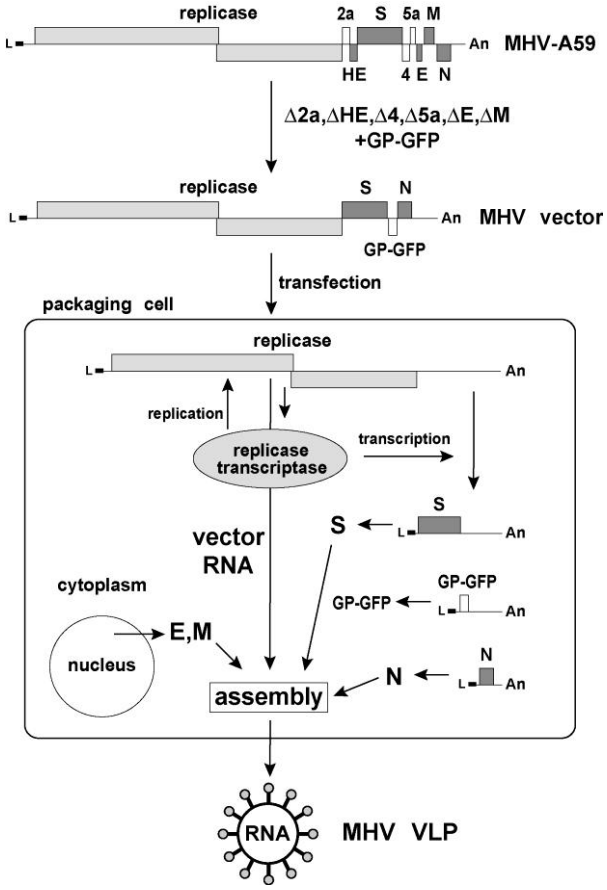

Figure 4: Overall strategy for the generation of MHV-GPΔEM vector and the packaging concept. Schematic representation of the initial design of MHV-GPΔEM vector and the corresponding packaging concept. The structural relationship and the genome organization of the MHV-A59 and the MHV-GPΔEM vector are shown. ORF1a and ORF1b encoding the replicase are depicted as light grey boxes, nonessential genes are indicated in white boxes and the structural genes are in dark grey boxes. Vector-encoded GP-GFP gene is indicated as white box. The bottom panel depicts the packaging concept. The structural proteins E and M are provided by the packaging cell in trans. The various vector-specific subgenomic mRNAs are depicted with a common 5' leader (L) sequence and a common 3' polyA tail. Structural proteins expressed by the MHV-based vector (S and N) or the packaging cell (E and M) will finally encapsidate the full-length vector RNA and assemble into virus-like particles that are released from the packaging cell.

The GP-GFP gene is cloned downstream of the TRS of MHV gene4 to enable transcription of a corresponding GP-GFP subgenomic mRNA. The construction of the MHV-GP-ΔEM vector was facilitated by the reverse genetic system established in our laboratory as described in material and methods (Coley et al., 2005).

In order to propagate MHV-GP-ΔEM vector, we have previously established a packaging cell line (17Cl1-EM-13) that stably express MHV E and M (Eriksson 2006). Although we could rescue MHV-GP-ΔEM on 17Cl1-EM-13 cells, we were unable to obtain vector stocks exceeding 10^3 VLPs/ml.

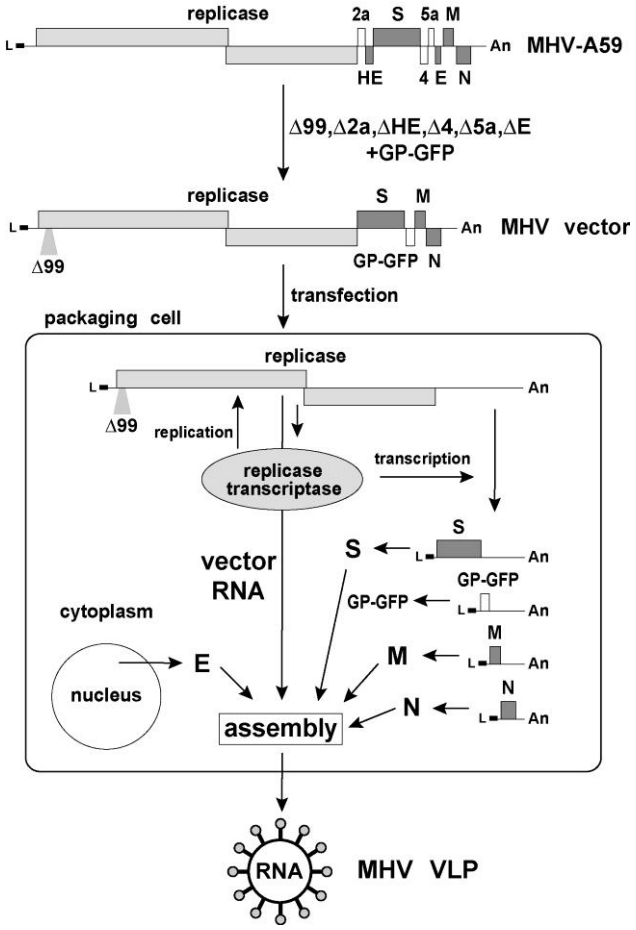

Figure 5: Overall strategy for the generation of MHV-GP vectors and the packaging concept. The structural relationship and the genome organization of the MHV-A59 and the MHV-GP vector are shown. MHV-A59 and MHV-GP vector encoded ORFs are depicted as boxes (figure1). Note that the MHV vector does encode the structural protein M but is devoid of E. Accordingly, the packaging cells do encode only E. Also note that the replicase encoded nsp1 in the MHV vector harbours an attenuating 99 nucleotide deletion.

In order to achieve high titer production of VLPs we revised our vector design reasoning that most likely, MHV-M protein expression in 17Cl1-EM-13 cells did not approach high expression levels observed in wild-type MHV-A59 infection. Therefore, we constructed MHV-vectors that lack only one structural gene, namely the E gene (figure 2). In contrast to the abundant expression of M protein, the expression of E protein is much lower in MHV-A59 infected cells. To secure highest biosafety, we deleted 99 nucleotides near the carboxy terminus of the replicase-encoded non-structural protein1 (nsp1). This deletion has been shown to provide attenuation in the context of a MHV-A59 mutant (Zust et al., 2007). In order to additionally assess a potential immunostimulatory effect of a cytokine, we cloned in one of the vectors between the replicase gene and the spike gene, the cytokine murine granulocyte colony stimulating factor (mGM-CSF). The resulting ΔE MHV vectors, designated MHV-GP and MHV-GM/GP, were initially rescued by electroporation of the full-length vector RNA into 17Cl1-EM-13 packaging cells. In contrast to the previously used ΔEM vectors, we could observe improved spread of MHV-GP and MHV-GM/GP vectors and reached peak titers of 10^4 VLPs/ml, suggesting that re-introducing the M gene into the vectors improved their propagation using the 17Cl1-EM-13 packaging cells.

Generation of an inducible MHV E expressing cell line

The ability to effectively evaluate MHV-based vectors for their suitability as vaccine vectors depends heavily on the availability of high titer vector stocks. According to our revised vector design, we aimed to generate an inproved packaging cell line that will only express the MHV-E protein and that will allow more efficient propagation of MHV-based ΔE vectors. We decided to use the Tet-Off system that allows for controlled inducible gene expression. Therefore, murine 17Cl1 and L929 cells that are susceptible to MHV infection were transfected with the regulator plasmid (pTet-Off vector) and a total of 61 17Cl1-based and 40 L929-based G418 resistant clones were selected. These clones were screened by transient transfection with pTRE2hyg-Luc in order to identify clones with low background and high induction of luciferase activity in response to the removal of tetracycline or its derivative doxycycline. All L929 cell clones indicated very low luciferase activity (data not shown) and therefore were discontinued. In contrast, most 17clone1-based Tet-Off cell lines displayed high luciferase expression (figure 3a). In parallel, we also assessed the susceptibility of these cell lines to MHV infection. In particular, we assessed MHV-A59 spread, plaque size and titers following MHV-A59 infection

of these cells (data not shown). These analyses resulted in the selection of clone number 17 for subsequent, stable introduction of the MHV-E expression plasmid pTRE2hyg-MHV-E.

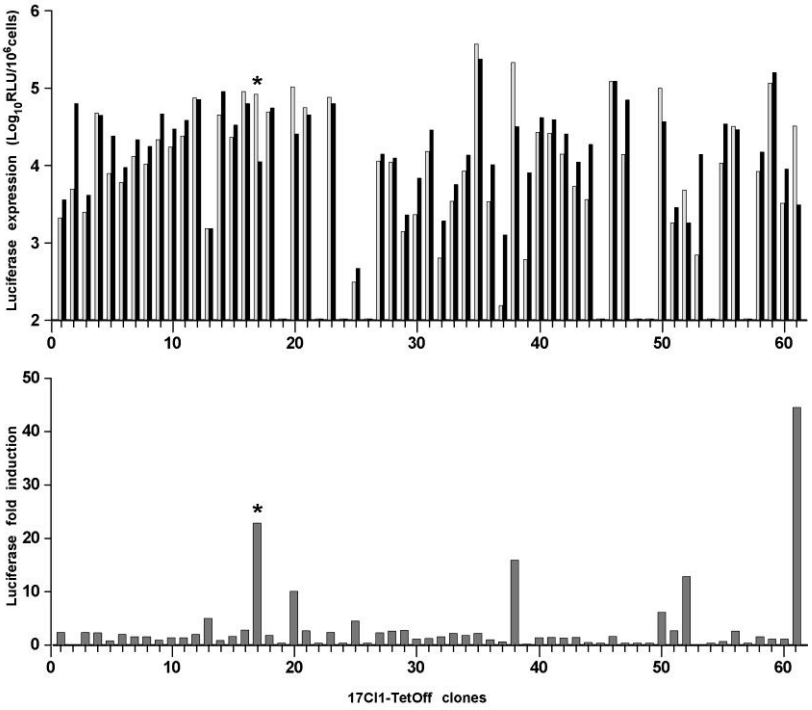

Figure 6. Development of an inducible packaging cell line based on the Tet-Off system. (A) Luciferase expression of 61 G418 resistant 17clone1-derived Tet-Off clones. Black bars represent luciferase expression under non-induced (+ Doxycycline) and white bars represent luciferase expression under induced (- Doxycycline) conditions. (B) Calculated fold induction of luciferase expression based on data shown in panel A. (*) depicts clone 17 that was selected for further usage.

After transfection of pTRE2hyg-MHV-E into clone number 17 and selection for hygromycin resistance, a total of 38 hygromycin resistant clones were isolated. These clones were evaluated for the genomic integration of the MHV-E by PCR. All 38 clones were positive by PCR for the genomic integration of MHV-E (data not shown). All clones were transduced with MHV-GP supernatant at a m.o.i. of 0.001 and the ease of MHV-GP spreading was carefully monitored (figure 4a), while supernatant was compared by titration on L929 cells to determine the best MHV-GP vector producing clone in terms of titer (figure 4b). Though most of the clones were very close to each other in MHV-GP spread and titers, subtle differences led to the selection of clones 3 and 20 (designated 17ECl3 and 17ECl20, respectively) to represent the best packaging cell clones with respect to MHV-GP production. Notably, the improved E-expressing

Tet-off-based cell lines greatly facilitated vector spread, propagation and we could obtain peak titers of up to 10^6 VLPs/ml (figure 4b).

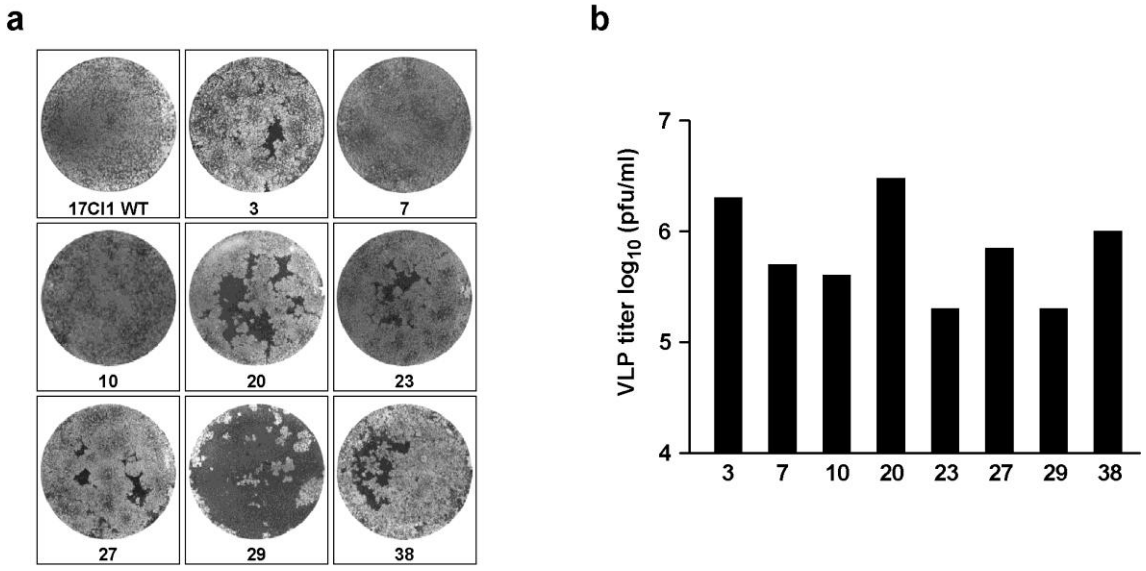

Figure 7. Evaluation and selection of clone 17 E expressing cell clones. Tet-Off derived clone 17 was transfected with pTRE2hyg-MHV-E. Expanded E expressing cell clones were transduced with MHV-GP vector particles and evaluated on the basis of VLP spreading and titer. (A) Indicate VLP spreading in various cell clones. (B) Indicate the titer of VLP obtained from the supernatant of the E expressing clones.

Generation of high titer VLP stocks.

Efficient analyses and characterization of MHV-based vaccine vectors requires sufficient amount of particles and in reasonable concentration as to be possible for mass immunization. Therefore, we aimed to optimize the MHV vector growth conditions using the 17ECl20 packaging cell line. When we transduced 1 x 10^6 17ECl20 packaging cells in a 6 well format with an m.o.i. of 0.001-0.01, at 33°C with low pH DMEM, we obtained titers of 1-3 x 10^6pfu/ml at 72 hrs post transduction. With similar m.o.i in 150 cm^2 flasks in a total volume of 20-30ml, we could also obtain titers of 1-3 x 10^6pfu/ml at 72hrs post transduction, suggesting that the packaging strategy is also applicable to the generation of MHV vectors in a larger scale. When we performed a similar experiment employing roller bottle amplification with m.o.i. 0.001-0.01, at 33°C low pH DMEM total volume of 30ml, we obtained titers of 5 x 10^6pfu/ml at 54hrs post transduction. Noteworthy, these titers obtained in roller bottles required less seed vector (lower moi) and less cell culture medium (30ml/10^8cells). In order to further increase VLP stock titers that could be used for mass immunization, we concentrated 100 ml of a 5 x 10^6 pfu/ml VLP

supernatant using PEG-6000 as described in methods. Using this procedure, it was possible to concentrate the VLP stocks to obtain titers of 10^7 - 10^8 pfu/ml.

Stability and safety.

After having established a robust packaging system to obtain high titer stocks, we aimed to assess if final MHV vectors stocks that were planed to enter immunization studies retained their integrity. First, we analyzed by RT-PCR if MHV-based vectors have retained the cloned antigen and cytokine coding regions. As shown in figure 5a, RT-PCR analysis using RNA from vector-transduced packaging cells revealed that both, the mGM-CSF- and the GP-GFP-coding regions were stably maintained. Furthermore, sequencing analysis of the resulting PCR products revealed that these regions were also stably maintained on the nucleotide level (data not shown). Second, we assessed whether MHV-based vectors may have undergone a potential recombination with the MHV-E mRNA provided in trans by the 17ECl20 packaging cells during rescue and propagation. As shown in figure 5b, E gene-specific RT-PCR using RNA obtained from MHV-GM/GP vector-transduced 17ECl20 packaging cells (lane 1), from non-transduced 17ECl20 packaging cells (lane 2), from MHV-GM/GP vector-transduced 17Cl1 cells (lane 3), from non-transduced 17Cl1 cells (lane 4), and from MHV-infected 17Cl1 cells (lane 5) revealed that E gene-specific RNA is detectable from packaging cells (due to stable expression in trans) and from MHV, but not from the MHV-GM/GP vector. Collectively, we conclude that MHV ΔE vectors grown to high titer stocks on 17ECl20 packaging stably maintain the cloned heterologous genes and did not acquire the E gene from packaging cells during propagation.

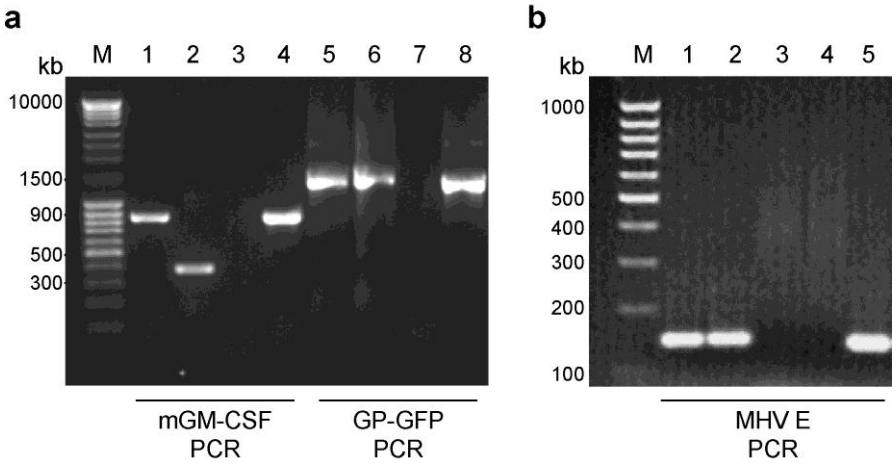

Figure 5. Analysis of MHV vector integrity. (A) RT-PCR analysis of MHV vector-encoded regions containing mGM-CSF (lanes 1-4) and GP-GFP (lanes 5-8) genes. RNAs were derived from 17ECl20 packaging cells

transduced with MHV-GM/GP (lanes 1 and 5) or MHV-GP (lanes 2 and 6). Lanes 3 and 7, water controls. Lanes 4 and 8, plasmid DNA controls. (B) MHV E gene-specific RT-PCR analysis. RNAs were derived from MHV-GM/GP-transduced 17ECl20 packaging cells (lane 1), 17ECl20 packaging cells (lane 2), MHV-GM/GP-transduced 17Cl1 cells (lane 3), 17Cl1 cells (lane 4), and MHV-infected 17Cl1 cells. M, DNA size marker.

Vector mediated-heterologous gene expression.

The rationale of an MHV-based vector is to mediate heterologous gene expression in specific target cells in order to induce a potent immune response. Therefore, to assess MHV-vector-mediated model antigen expression, we transduced bone marrow derived dendritic cells with MHV-GM/GP and MHV-GP (m.o.i=1), and 12 hrs post transduction, cells were analyzed by fluorescence activated cell sorting (FACS) for green fluorescence protein expression. As shown in figure 6a, MHV vector-mediated GFP expression was seen in both MHV-GM/GP and MHV-GP transduced DCs. Notably, we observed a higher proportion of surviving DCs and a higher proportion of green fluorescent DCs when the mGM-CSF-expressing vector MHV-GM/GP was used for transduction. In order to assess vector-mediated mGM-CSF expression, 17ECl20 packaging cells, bone-marrow derived DCs and peritoneal macrophages were transduced at an m.o.i of 0.01 with MHV-GM/GP and incubated at 37°C for 48hrs during which supernatant was collected at the indicated time points (figure 6b). We observed efficient vector-mediated expression of mGM-CSF in 17ECl20 packaging cells, and more importantly, also in primary target cells such as DCs and macrophages (figure 6b). Taken together, these data demonstrate that it is possible to clone a particular antigen in the MHV-vector genome and target it to specific immune components capable of stimulating a potent immune response.

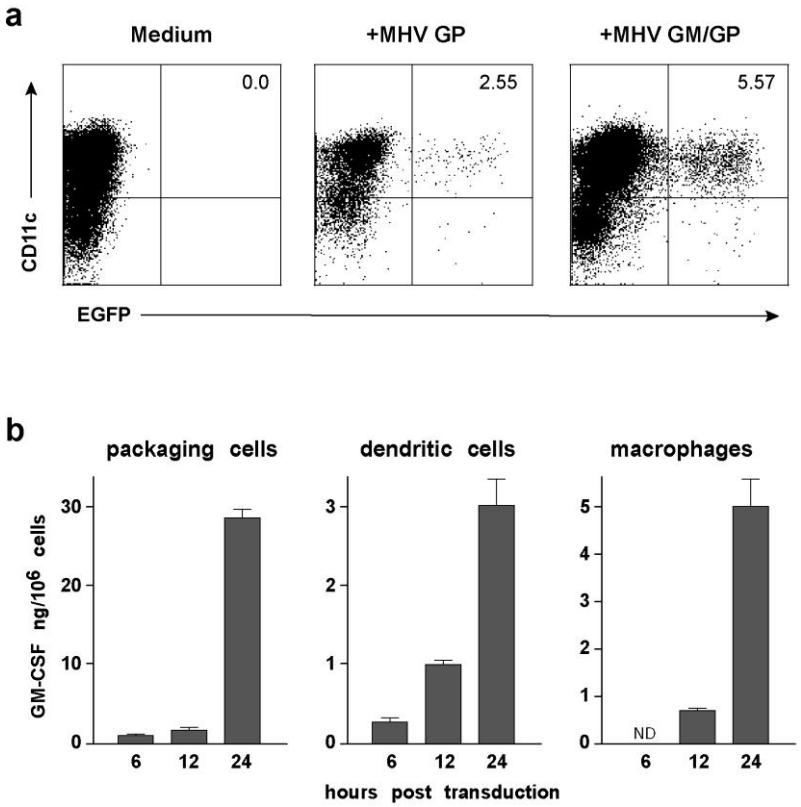

Figure 8. MHV vector-mediated heterologous protein expression in vector-transduced cells. (A) FACS analysis of GFP expression in $CD11c^+$ murine bone marrow derived DCs 12 h post transduction with MHV-GP (middle panel) or MHV-GM/GP (right panel) or mock transduced (left panel). (B) mGM-CSF expression in MHV-GM/GP transduced (m.o.i=0.01) 17ECl20 packaging cells (left panel), $CD11c^+$ murine bone marrow derived DCs (middle panel), and murine peritoneal macrophages (right panel). Data were assessed by using mGM-CSF ELISA at the indicated time points post transduction.

4. **Discussion**

We report here the design, development and characterization of the first murine coronavirus-based multigene RNA vector particles. MHV-based vectors have several inherent advantages that make them attractive as vaccine vector candidates. We have constructed a set of prototype MHV-based vectors that can mediate model antigen and cytokine expression simultaneously. Furthermore, we demonstrate here that MHV-based vector RNAs were stably maintained upon propagation and that high titer VLP stocks can be prepared using an appropriate packaging concept and improved growth conditions.

In order to construct these MHV-based vectors we exploited the recombinant vaccinia virus vMHV-inf-1 carrying the full length cDNA of MHV-A59 (Coley et al., 2005) using the reverse genetic system established in our laboratory. By using genetically tailored recombination cassettes we systematically replaced the genes encoding the MHV ORF4, ORF5a and the gene encoding the small envelope protein E with the *E. coli*-gpt gene to enable selection of desired clones. In the second step we have introduced an expression cassette carrying the GP-GFP gene cloned downstream of MHV TRS so as to assure adequate transcription of these antigens. In addition, we inserted a cytokine gene, the murine GM-CSF gene, in the region between the MHV replicase and the MHV spike. The GM-CSF gene was cloned downstream the MHV TRS-2 to ensure the production of a vector-derived subgenomic mRNA.

In order to rescue and propagate MHV-based vectors it was necessary to produce a stable packaging cell line that will provide the deleted MHV structural proteins E and M in trans. In our first design (figure 4) where we aimed at a one hit vector system by deleting the MHV structural proteins E and M, we initially masterminded a packaging concept and developed a packaging cell line expressing MHV E and M in trans (figure 4). Rescue of the MHV-GP-ΔEM vector was successful using this cell line but we were unable to efficiently propagate this vector using the initial packaging strategy. One obvious reason for this observation is that the amount of M protein that was provided by the cells in trans was not sufficient to enable efficient propagation of the MHV-GP-ΔEM vector.

The safety of coronaviruses as vectors has been demonstrated by several groups based on the deletion of the small envelope gene E (Curtis et al., 2002; Ortego et al., 2002). Recently, a coronavirus-based vaccine, a ΔE-SARS vaccine has been reported to confer immunogenicity and protective efficacy in hamster (Lamirande et al., 2008). In fact, the lack of the E gene in TGEV greatly affects maturation of progeny virion in the secretory pathway indicating the importance of this gene for TGEV (Ortego et al., 2007). In contrast, the MHV-E gene has been shown not to

be absolutely necessary for replication and MHV-ΔE has been demonstrated to produce tiny plaques, exhibit a low growth rate as well as yield low infectious titers when compared to the wild-type MHV (Kuo and Masters, 2003). Therefore, in the revised design of our vectors (figure 5) we have deleted the MHV-A59 E gene in order to attenuate our vectors. Interestingly, it has been elegantly reported that coronavirus group specific genes are not essential for their replication in tissue culture but that their deletion is attenuating in the natural host (de Haan et al., 2002a; Haijema et al., 2004), therefore, we reasoned that, deleting the nonessential genes of MHV-A59 will provide two advantages (i) further attenuate these vectors and (ii) increase the capacity for heterologous antigen accommodation. In fact one way of attenuating a virulent virus is by knocking out a possible pathogenicity factor. SARS nsp1 has been shown to interfere with the first line of antiviral defence (Kamitani et al., 2006; Narayanan et al., 2008; Wathelet et al., 2007) and a mutant SARS nsp1 virus was shown to be attenuated in interferon competent cells (Wathelet et al., 2007). Similarly, the disruption of MHV-A59 nsp1 has been shown not to affect the replication of the virus in interferon incompetent mice whereas in interferon competent animals, this hepatotropic virus was highly attenuated (Zust et al., 2007). Therefore, we included the disruption of the MHV nsp1 in our vector design in order to ensure highest biosafety. Taken together, these vectors devoid of all nonessential proteins, encoding a 99-nucleotides deletion in the replicase encoded nsp-1 region and lacking the structural protein E are considered to be highly biosafe vaccine vectors.

Furthermore, we resorted to develop a new packaging concept based on the Tet-Off inducible system. This system provides the added advantage that we can regulate the expression of the trans gene therefore indirectly influencing the packaging outcome. More so the decision of Tet-Off as against Tet-On is backed by the fact that we avoid antibiotics into our vaccine vector stocks. In our revised packaging concept, therefore, we developed an inducible Tet-Off cell line that provides the MHV-E protein in trans. We initially transfected 17clone1 based cells with the Tet regulator plasmid and selected 61 G418 resistant clones. These clones were screened after transient transfection with a luciferase control plasmid for luciferase activity. Following a calculated fold of luciferase induction and infectivity screening, the clone number 17 was selected. This clone was transfected with a plasmid encoding the MHV-E and 38 E expressing hygromycin resistant clones were selected. These cell lines were screened by transduction with vector particles at very low multiplicity of infection and the ease of vector spreading was monitored. The titer of the supernatant obtained from those clones led to the selection of clone 3 and 20 to represent the best vector producing cell lines. The use of Tet-Off-based E expressing packaging cell line 17ECl20 enabled us to robustly propagate the ΔE MHV vectors.

In order to amplify the VLP stocks we employed a systematic upgrading of the stocks by first transducing 150cm^2 tissue culture flaks and after obtaining the supernatant in sufficient quantity, we went on to transduce the roller bottle. We obtained titers of 5x10^{6}pfu/ml. Furthermore, we could concentrate the VLP stocks by a coupling reaction with polyethylene glycol-6000 to titers of 10^{8}pfu/ml. This therefore demonstrates our determined stepwise improvement of the VLPs stocks to such a high amount that can be employed in nearly any kind of vaccination schedule.

Furthermore, the stability of MHV-based vectors was analyzed after 12 passages by RT-PCR and we observed that these vectors are stable after 12 passages and this was confirmed by sequencing analyses. The vectors were equally checked for any sign of recombination with the MHV-E gene that is provided in the packaging cell line. The RT-PCR results, indicate that the vectors remain safe despite their extensive propagation on E producing packaging cells.

The main goal of vaccine vectors is to mediate expression of their genetic cargo in specific target cells or tissue. Expression of multiple heterologous proteins in the context of a prototype vector expressing three cytoplasmic reporter markers has been described (Thiel et al., 2003) for HCoV 299E. When we transduced murine bone marrow derived DCs with MHV-GM/GP and MHV-GP vectors, we observed the expression of GP-GFP in these target cells. The heightened stringency on vaccine safety has plunged new vaccines into a situation that is often linked to lower immunogenicity compared to previous whole cell or virus-based vaccines. This calls for the necessity to employ adjuvant to induce potent and durable immune responses with additional benefit that less antigens and fewer injections may be needed (Guy, 2007). Currently, there is an evaluation spree for cytokines as adjuvants in vaccines as against most chemical entities because cytokines are likely to be the direct proximal mediators of the ‘classical’ adjuvants (Pashine et al., 2005). In our design we have considered the possibility to manipulate the immune outcome after vaccination with our vectors therefore we cloned a cytokine the mGM-CSF in one of the vectors. In deed, we observed better recovery of GP-GFP positive cells with a percentage of 5.57 as compared to the vector without GM-CSF (2.55%). This result indicates that the GM-CSF encoding vector allows better survival of the target cells as compared to the non-GM-CSF vector. Additionally, this may have an implication in the immune outcome. In fact, dendritic cells and macrophages represent important target cells in terms of a vaccine vector since these cells can have important influence on the outcome of an immune response. Taken together, this result demonstrates efficient vector-mediated expression of heterologous genes in the context of model antigens. Overall, these experimental results provide the proof of principle that the generation of MHV-based vectors is feasible and that the transgenes expressed

by these replication competent but propagation deficient vectors can be targeted to DCs and macrophages.

With the vector system established in this study it is now possible to systematically assess the efficacy of this novel vaccine platform. Specifically we will study the effect of mGM-CSF on DC activation and maturation. Furthermore, we will assess the immunogenicity of the MHV vectors in mice using immunization strategies against pathogenic viral challenge and experimental tumours expressing the relevant model tumour or viral antigen.

It is worth noting that mouse hepatitis virus is one of the best studied viruses in the laboratory and this comes in hand with the availability of a variety of well characterized small animal models permitting the effective evaluation of the system using well established and generally recognised scientific procedures. Hence the murine model of coronavirus-based vectors represent a very important tool for the development and evaluation of the system and may be instructive for the development, evaluation and adaptation of coronavirus-based vaccine vectors for human ailments (Eriksson et al., 2006b).

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6.2 Dendritic cell-targeting through coronavirus-based vaccine vectors induces long-lasting protective antiviral and antitumour immunity

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Abstract

Coronaviruses are large positive-stranded RNA viruses that can be exploited to genetically deliver multiple antigens and immunostimulatory cytokines to professional antigen-presenting cells. Here, we report the assessment of the immunogenic potential of coronavirus-based vectors in a murine model. We have constructed a set of bio-safe vaccine vectors based on mouse hepatitis virus (MHV) genomes that are devoid of accessory genes, lack one structural gene, and carry an attenuating deletion in the replicase gene. They have been designed to form virus-like particles that mediate the expression of the lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) or human melan-A CTL epitopes in combination with the immunostimulatory cytokine GM-CSF. We demonstrate selective targeting of dendritic cells (DCs) and macrophages resulting in vector-mediated antigen expression in vitro and in vivo, and efficient GM-CSF-mediated maturation of DCs. In mice, single immunization elicited strong and long-lasting protective cytotoxic T-cell responses against challenge with LCMV and LCMV-GP recombinant vaccinia virus. Furthermore, single dose application of the vaccine elicited both prophylactic and therapeutic immunity against metastatic melanoma. In summary, this novel vaccine platform mediates the delivery of antigens and immunostimulatory cytokines to the cellular components of the immune system that initiate and maintain protective immunity.

Introduction

Vaccination against viral infections has saved millions of lives by protecting many individuals from diseases such as measles, rubella, mumps, and polio. However, there is growing need to develop not only improved vaccines against acute infections but also to generate therapeutic vaccines which may stimulate specific immune response to persistent viruses such as the human immunodeficiency virus or the human hepatitis C virus (McMichael, 2006; Strickland et al., 2008). Likewise, novel approaches for vaccination against tumours are needed which counteract the immunosuppression associated with cancer (Melief, 2008). There is compelling evidence that $CD8^+$ cytotoxic T cells are crucial players in the protective immune response against viral infections, and tumours (Appay et al., 2008). Novel vaccine approaches should thus be rigorously evaluated for their ability to maximally expand antigen-specific $CD8^+$ T cells, to induce their optimal differentiation into effector $CD8^+$ T cells, and to elicit long-lasting protective memory (Appay et al., 2008).

A major bottle-neck in the development of new and effective vaccines is the delivery of antigens to dendritic cells (DCs) (Steinman and Banchereau, 2007; Tacken et al., 2007) which sample antigen, transport immunogenic components to secondary lymphoid organs, and initiate and maintain T and B cell responses. The excellent capacity of adoptively transferred DCs to prime antiviral T cell responses can be readily shown in vivo; only 100 – 1000 DCs presenting a specific viral antigen have to reach secondary lymphoid organs for the induction of protective antiviral T cell responses (Ludewig et al., 1998; Ludewig et al., 2000a). DC-induced CTL responses develop rapidly and DC-immunized mice maintain robust memory T cell responses which protect against systemic and peripheral viral challenge (Ludewig et al., 1999). Likewise, several preclinical studies demonstrated that efficient antitumour immunity can be induced using adoptive transfer of DCs (Ludewig et al., 2000b; Nair et al., 1998; Ochsenbein et al., 1999; Rea et al., 2001a; Song et al., 1997; Specht et al., 1997). Although individualized adoptive transfer of antigen-loaded DCs is feasible and – to a certain extent – efficient in clinical application in humans (Palucka et al., 2007), off-the-shelf vaccines that permit targeted delivery of antigens to DCs in vivo are certainly advantageous and will eventually find their way into clinical application.

The description of cell-surface molecules that exhibit a rather specific expression on DCs has fueled the development of antibody-based targeting strategies (Bonifaz et al., 2004; Bozzacco et al., 2007; Meyer-Wentrup et al., 2008; Tacken et al., 2005). Although these protein-based vaccines generate $CD4^+$ T cell and B cell responses against a range of different antigens,

antigen-coupling to antibodies exhibits a major limitation for the induction of $CD8^+$ T cell response, that is the strict dependence on cross-presentation (Steinman, 2008; Tacken et al., 2007). Viral vectors encoding for immunogenic antigens can deliver their genetic cargo directly into DCs thus generating antigenic peptides in the infected cell and allowing for efficient loading of MHC class I molecules. Among the currently most exploited viral vectors that facilitate antigen delivery to DCs are adenoviral (Cheng et al., 2007; Rea et al., 2001b), and lentiviral vectors (Ageichik et al., 2008). However, one major impediment of these vectors is the frequent off-target transduction resulting in antigen presentation by parenchymal cells outside secondary lymphoid organs. For example, the strong tropism of adenoviral vectors for hepatocytes with deposition of >95% of the genetic material in the liver, leads to generation of functionally impaired $CD8^+$ T cells (Krebs et al., 2005; Yang et al., 2006). Major efforts are thus required to engineer adenoviral vectors with improved specificity for the relevant antigen presenting cells (Cheng et al., 2007; Rea et al., 2001a). Likewise, lentiviral vectors preferentially infect cells other than DCs and re-direction of their target cell tropism is warranted (Lopes et al., 2008; Yang et al., 2008). A second potential impediment for the use of this class of viral vectors in the clinics is their potential to integrate genomic material into the host genome (Dobbelstein, 2003).

Coronaviral vectors display a number of features that clearly overcome these limitations: (i) Replication of these positive-stranded RNA viruses occurs in the cytoplasm without a DNA intermediary, making insertion of viral sequences into the host cell genome unlikely. (ii) The large RNA genomes provide a cloning capacity of more than 6 kb. (iii) The unique transcription process generates 6-8 subgenomic mRNAs encoding mainly for the structural genes which can be replaced by multiple heterologous genes (Thiel et al., 2003). (iv) Receptors of human and murine coronaviruses (e.g. human coronavirus (HCoV 229E, or MHV) are expressed on human and murine DCs, respectively (Summers et al., 2001; Zhou and Perlman, 2006) .

We describe here the generation and evaluation of inherently safe coronavirus-based viral vectors that efficiently target antigens and immunostimulatory molecules to DCs. We show that MHV-based vectors can deliver multiple antigens and immunostimulatory cytokines almost exclusively to $CD11c^+$ DCs within secondary lymphoid organs. Delivery of only few viral particles elicited potent $CD8^+$ T cell responses that provided long-lasting protection against viral challenge. Moreover, a single application of the novel viral vectors generated prophylactic and therapeutic immunity against metastatic melanoma. Induction of CTL directed against the human Melan-A antigen in HLA-A2 transgenic mice indicates that coronavirus-mediated gene transfer to DCs represents a versatile approach for immunization against both viral infection and cancer.

Material and Methods

Mice and cells. C57BL/6 mice were obtained from Charles River Laboratories (Sulzfeld, Germany). A2DR1 have been kindly supplied by Dr. Lemonnier (Pasteur Institute, Paris) (Pajot et al., 2004). All mice were maintained in individually ventilated cages and were used between 6 and 9 weeks of age. All animal experiments were performed in accordance with the Swiss Federal legislation on animal protection. L929, and CV-1 cells were purchased from the European Collection of Cell Cultures. MC57 and BSC40 cells were obtained from Dr. Zinkernagel (University of Zürich, Switzerland). D980R cells were a kind gift from G. L. Smith, Imperial College, London, UK. 17Clone1 cells were a kind gift from S.G. Sawicki, Medical University of Ohio, Toledo, Ohio, USA. BHK-MHV-N cells, expressing the MHV-A59 nucleocapsid protein under the control of the TET/ON system (Clontech), have been described previously (Coley et al., 2005).

Production of replication-deficient, propagation-competent MHV particles. Mutant vaccinia viruses are based on the recombinant vaccinia virus vMHV-inf-1 (containing the full length MHV-A59 cDNA) and were generated using the reverse genetic system established in our laboratory as described previously (Coley et al., 2005; Eriksson et al., 2008). To construct the recombinant vaccinia virus vMHV-GP the MHV-Inf-1 was modified by replacing the MHV nucleotides (nts) 21771 – 29623 by gpt using the plasmid pMHV-rec-1. This plasmid carries the *E. coli*- gpt gene flanked to its 5'end by a sequence corresponding to MHV nts 21328 – 21771 and to the 3'end by a sequence corresponding to MHV nts 29655 – 30114. The resulting gpt positive vaccinia virus clone vMHV-1b-gpt-N was used to clone the MHV-GP vector cDNA by inserting the MHV spike gene, the GP-GFP gene and the MHV-M and N genes in a second round of recombination with the plasmid pMHV-vec-10-ΔE. This plasmid was composed of 5'-MHV nts 21328 – 21771, MHV nts 23930 – 27964, the GP-GFP gene, MHV 28969 – 30114-3'. The resulting vaccinia virus-vMHV-GP was used to rescue the MHV vector MHV-GP.

To construct the recombinant vaccinia virus vMHV-GM/GP the gene encoding the murine granulocyte colony stimulating factor (mGM-CSF) was cloned between the MHV replicase and spike genes. First, the recombinant vaccinia virus vMHV-GP was modified using the plasmid pMHV-rec-2-gpt composed of 5' MHV nts 21328 – 21771, the *E. coli*-gpt-gene, and MHV nts 23890 – 24452. The resulting gpt-positive clone vMHV-1b-gpt-S-GP was modified in a second round of recombination with the plasmid pMHV-rec-2-mGM-CSF composed of 5' MHV nts 21328 – 21771, the mGM-CSF gene, MHV nts 23890 – 24452 and a gpt negative

selection was performed. The resulting vaccinia virus vMHV-GMGP was used to rescue the vector MHV-GM/GP.

To construct the recombinant vaccinia viruses vMHV-MelA and vMHV-GM/MelA, the vaccinia viruses vMHV-GP and vMHV-GM/GP were modified to replace the GP-GFP gene by the gene encoding a fusion protein MelA comprised of the GFP-coding region, the yeast ubiquitin-coding region and the Mel-A_{26-35}A27L analog peptide derived from the human Melan-A/MART-1 protein (Valmori et al., 1999). First, plasmid pMHV-rec3 was used to recombine with vMHV-GP and vMHV-GM/GP. pMHV-rec3 is composed of MHV-nts 27386 – 27964, the *E. coli*-gpt-gene, and MHV nts 28929 – 29655. The resulting recombinant vaccinia viruses vMHV-S-gpt-M and vMHV-GM-S-gpt-M were recombined with plasmid MHV-rec3-MelA composed of MHV-nts 27386 – 27964, the MelA gene, and MHV nts 28929 – 29655. The resulting in the recombinant vaccinia viruses vMHV-MelA and vMHV-GM/MelA were used to rescue the vectors MHV-MelA and MHV-GM/MelA, respectively.

The generation of the Tet-Off expression system-based (Clontech) MHV vector packaging cell line 17ECl20 was done as follows. First, murine 17Cl1 cell were stably transduced with the plasmid pTet-Off-vector and subjected to G418 selection as recommended by the manufacturer (Clontech). A total number of 61 G418-resintant clones were screened for firefly luciferase expression under induced and non-induced conditions and clone number 20 was selected for the introduction of the plasmid pTRE2hyg-MHV-E. This plasmid is based on the pTRE2hyg-vector plasmid (Clontech) into which the MHV-E gene was cloned into the BamHI and NotI sites of the multiple cloning site. The MHV-E gene was obtained by PCR using the primers: 5'-ACTGGGATCCACCATGTTTAATTTATTCCTTACAG-3' reverse 5'-ACTGGCGGCCGCTTAGATATCATCCACCTCTAATAG-3' (BamHI and NotI sites underlined). Following hygromycin selection a total number of 38 hygromycin-resisitant clones were analyzed for MHV-E gene incorporation by PCR and assayed for optimal MHV vector propagation. The cell clone revealing highest MHV vector titers, designated 17ECl20 was then used for MHV vector propagation and high titer stock preparation.

Recombinant MHV-based vectors were rescued from cloned cDNA using purified vaccinia virus DNA as template for in vitro transcription of recombinant MHV-based vector genomes as described (Eriksson et al., 2006; Eriksson et al., 2008). In order to rescue recombinant coronavirus-based vectors and package them into virus-like particles (VLPs), the full length vector RNA was transfected into 17ECl20 packaging cells by electroporation (Eriksson et al., 2008). The transfection is incubated in minimum essential medium supplemented with 5% Tet-approved FBS (Clontech), 500 U penicillin/ml, 100u

streptomycin/ml (Tet-induction MEM) at 37°C 5% CO_2 for about 2 days. MHV-based vector particles released into the supernatant within the next 24-48 hrs were collected and stored at -80°C until further usage. High titer MHV vector stocks were produced after transduction of 10^7 – 10^8 17ECl20 packaging cells with MHV vectors (moi=0.001 – 0.01). 100 ml of MHV vector supernatants from these cultures were subjected to polyethylenglycol (PEG) precipitation by adding 7.4 ml ice cold 5M NaCl, slow stirring at °C for 30 min, adding 54 ml 30% (w/v) PEG-6000 and centrifugation (15.000g, 4°C). The MHV vector pellet was resolved in 2 ml of MEM containing 2% FCS. Titration of MHV vectors was performed on murine L929 cells as described (Zust et al., 2008).

Determination of antiviral protection. LCMV-WE strain was obtained from Dr. R. Zinkernagel (Universität Zürich, Switzerland), propagated on L929 cells, and titered on MC-57 cells. Recombinant vaccinia virus expressing LCMV glycprotein (VV-G2) was originally obtained from Dr. D.H. Bishop (Oxford, U.K.), and recombinant VV expressing vesicular stomatitis virus Indiana glycoprotein (VV-INDG) was originally obtained from Dr. B. Moss (National Institutes of Health, Bethesda). Vaccinia viruses were grown and titrated on BSC40 cells.

To examine anti-viral protection, unless otherwise indicated naïve mice were immunized with 105pfu of MHV-GP or MHV-GM/GP diluted in cold BSS and 7 days post immunization (p.i), mice were challenged with 200 pfu LCMV-WE i.v. or 2 x 10^6 pfu of recombinant vaccinia virus encoding either the glycoprotein of LCMV-WE or of VSV intra-peritoneally (i.p.). Four days post challenge, mice were killed and virus titers in the spleens were determined by LCMV infectious focus assay on MC57 cells as previously described (Battegay et al., 1991) or five days post challenge, ovaries were collected and vaccinia virus plaque assay was performed as described (Eriksson et al., 2008).

Isolation of dendritic cells and macrophages, flow cytometry and antibodies. Bone marrow–derived cDCs were generated by 6 to 7 days of culture with granulocyte-monocyte colony stimulating factor (GM-CSF)-containing supernatant from the cell line X63-GM-CSF (kindly provided by Antonius Rolink, University of Basel, Basel, Switzerland). cDCs were further purified using Optiprep density gradient centrifugation. Thioglycolate-elicited macrophages were collected from the peritoneal cavity of mice and cultured overnight at 37°C. Non-adherent cells were removed by washing with ice-cold PBS. Splenic DCs were isolated from splenocyte suspensions of collagenase type II digested (Gibco, Invitrogen Basel, Switzerland) spleens. The

$CD11c^+$ DC fraction was enriched using mouse CD11c (N418) microbeads (Miltenyi Biotec) as recommended by the manufacturer.

Splenocytes were obtained from spleens of B6 following digestion with collagenase type II for 20 min at 37°C and resuspended in RPMI 5%. For isolation of the low density enriched population, cells were resuspended in PBS supplemented with 2% FCS, 2 mM EDTA and overlaid on 20% Optiprep density gradient medium (Sigma-Aldrich Co. Basel, Switzerland). After centrifugation at 700 × g for 15 min, low density cells were recovered from the interface and resuspended in RPMI 5%. Cells were stained with different lineage markers and analyzed for GFP expression with a FACSCalibur flow cytometer using the CellQuest software (BD Biosciences). Antibodies used in this study were purchased from BD Biosciences Pharmingen (CD11c-PE), Biolegend (CD19-PE, CD4-PE, B220-APC, CD3-APC, CD11b-APC, CD86 APC, CD40 Alexa 647), and eBiosciences (F4/80-PE, CD8-PE).

Tetramer analysis and intracellular cytokine staining. Specific ex vivo production of IFN-γ was determined by intracellular cytokine staining. Organs were removed at the indicated time points following infection with recombinant MHV. Tetramers were synthesized and applied for staining of blood and splenic samples as previously described (Junt et al., 2002; Valmori et al., 1999). For intracellular cytokine staining, single cell suspensions of 1×10^6 splenocytes were incubated for 5 h at 37°C in 96-well round-bottom plates in 200 µl culture medium containing 25 U/ml IL-2 and 5 µg/ml Brefeldin A (Sigma). Cells were stimulated with phorbolmyristateacetate (PMA, 50 ng/ml) and ionomycin (500 ng/ml) (both purchased from Sigma, Buchs, Switzerland) as positive control or left untreated as a negative control. For analysis of peptide-specific responses, cells were stimulated with 10^{-6} M of the indicated peptides. The percentage of $CD8^+$ T cells producing IFN-γ was determined using a FACSCalibur flow cytometer. GP33 (KAVYNFATC) and GP34 (AVYNFATC) peptides were purchased from Neosystem (Strasbourg, France). Melan-A peptide (ELAGIGILTV) was obtained from the Ludwig Institute for Cancer Research.

Melanoma model. B16F10-GP melanoma cells expressing the LCMV gp33 epitope (Prevost-Blondel et al., 1998) and parental B16F10 cells were kindly provided by Dr. H. Pircher (University of Freiburg, Germany). The B61F10-GP melanoma cells were cultivated under G418 (200 µg/ml) (Life Technologies, Gaithersburg, MD) selection. B6 mice received 5×10^5 tumour cells i.v. and numbers of lung metastasis were determined on day 12 post inoculation. For

tumour prevention experiments, B6 mice were immunized with MHV-based vectors at the indicated days before tumour challenge. Tumour therapy experiments were performed in an analogous fashion with except that MHV-GM/GP immunization was done on the same day as B16F10-GP melanoma cells were given or on day 4 or 8 after B16F10-GP melanoma cells were given.

Statistical analysis. All statistical analyses were performed with Prism 4.0 (Graphpad Software Inc.). Data were analyzed with the paired Student's t-test assuming that the values followed a Gaussian distribution. A P value of < 0.05 was considered as significant.

Results

Design and propagation of bio-safe coronavirus-based vaccine vectors.

To assess immunogenicity and to ensure maximal safety of coronavirus-based multi-gene vaccine vectors, we have rationally designed a series of prototype vectors based on the mouse hepatitis virus, strain A59 (MHV-A59) (Fig. 1). For the generation of attenuated and propagation-deficient MHV-based vectors, we have (i) deleted all MHV-encoded accessory genes (NS2, HE, gene4, gene5a) (de Haan et al., 2002), deleted 99 nucleotides within the replicase-encoded sequence of the non-structural protein 1 (nsp1) (Zust et al., 2007), and deleted the structural gene E (Ortego et al., 2002).

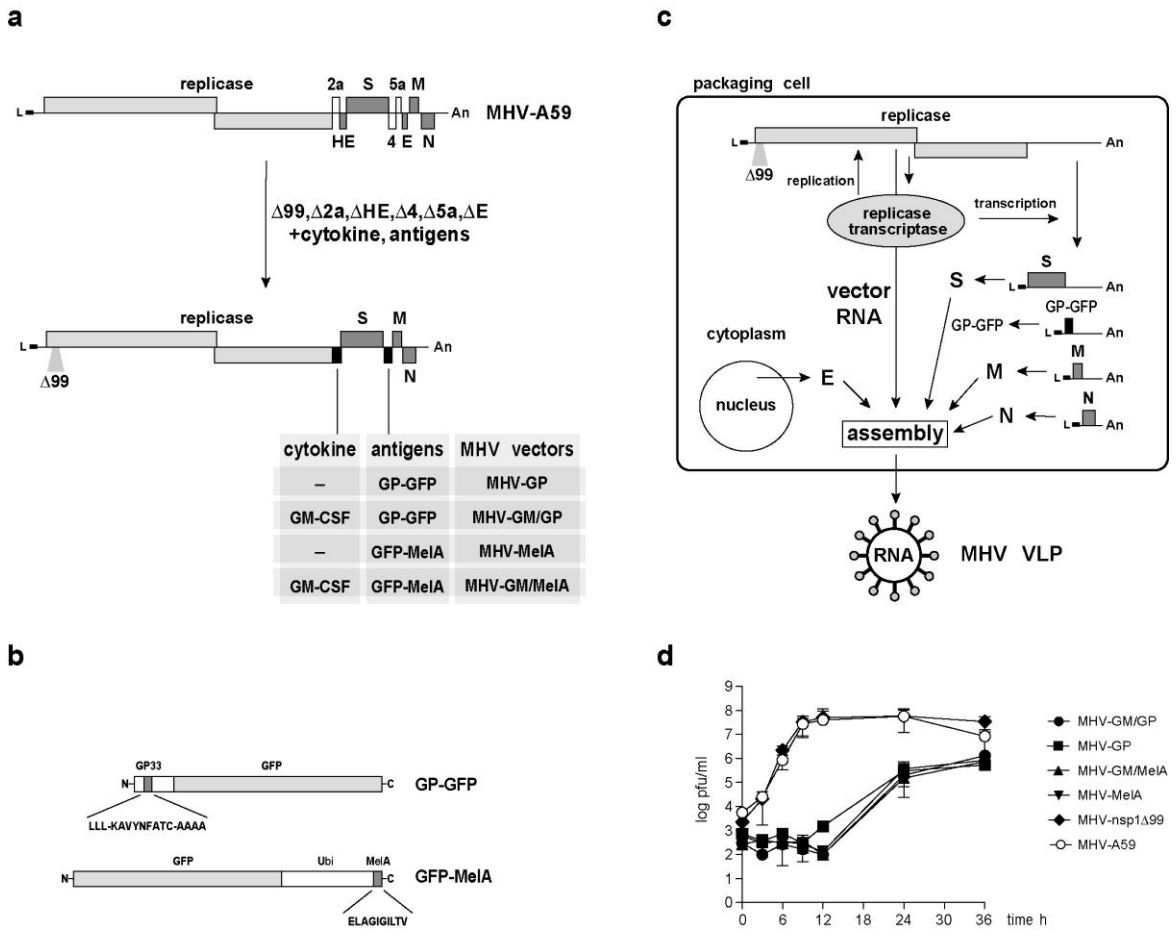

Figure 1. Generation and propagation of MHV-based vaccine vectors. (A) Schematic representation of MHV-A59 genome and the highly attenuated MHV vectors encoding different antigen cassettes and the immunostimulatory cytokine GM-CSF. (B) Depiction of the GFP-antigen fusion cassettes. (C) Packaging concept for the generation of replication-competent but propagation-deficient MHV particles. (D) Growth kinetics of the indicated MHV vectors, MHV-nsp1Δ99 and MHV-A59 in 17ECl20 packaging cells. Cells were infected at an moi of 1 and titers in supernatants were determined at the indicated time points.

In order to test the coronavirus-based vaccine concept and to develop a vaccine that provides strong CTL responses, both in terms of magnitude and longevity, we have used the CTL epitopes gp_{33-41} derived from the lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP), and Mel-A_{26-35}A27L analog peptide derived from the human Melan-A/MART-1 protein (Valmori et al., 1999) (Fig. 1a).

As shown in figure 1b, both CTL epitopes were cloned as fusion proteins with the green fluorescent protein (GFP) and the corresponding genes were cloned between the MHV-vector-encoded spike and membrane genes. Since maturation and appropriate stimulation of DCs is critical for the generation of efficient T cell responses (Steinman et al., 2003) and hence an indispensable component of rationally designed vaccines (Chabalgoity et al., 2007), we have inserted the gene encoding for the murine granulocyte-macrophage colony-stimulating factor (GM-CSF) between the MHV-vector-encoded replicase and spike genes (Fig. 1a).

In order to propagate the MHV-based vectors to high titers, we have produced a packaging cell line, designated 17ECl20, based on murine 17Cl1 cells that express the MHV E protein under the control of the Tet-Off system (Fig. 1c). As shown in figure 1d, all MHV-based vectors grew efficiently in 17ECl20 cells and reached peak titers of approximately 10^6 VLPs/ml and final MHV vectors stocks of 10^8 VLPs/ml were produced following polyethylenglycol precipitation.

DC and macrophage-specific delivery and expression of coronavirus vector-encoded antigens and cytokines

To assess MHV-vector growth kinetics on cells that do not express the MHV E protein, we transduced murine L929 cells, bone marrow-derived $CD11c^+$ DCs, or peritoneal macrophages with the MHV-vectors and compared growth kinetics to that of wild-type MHV-A59 and the nsp1 mutant MHV-nsp1Δ99. As shown in figure 2a, MHV-based vector growth was greatly impaired in murine L929 cells. Most importantly, in peritoneal macrophages and DCs, MHV-vector transduction did not result in VLP release, demonstrating that the lack of the E protein prevents MHV-vector propagation in primary cells.

All MHV vectors led to significant GFP-expression in transduced cells L929 cells and peritoneal macrophages (data not shown), and rapid production of GM-CSF in L929 cells, peritoneal macrophages and DCs following exposure to the cytokine-encoding vectors (Fig. 2b), demonstrating that MHV vectors can be used to simultaneously express heterologous antigens and immunostimmulatory cytokines in susceptible target cells.

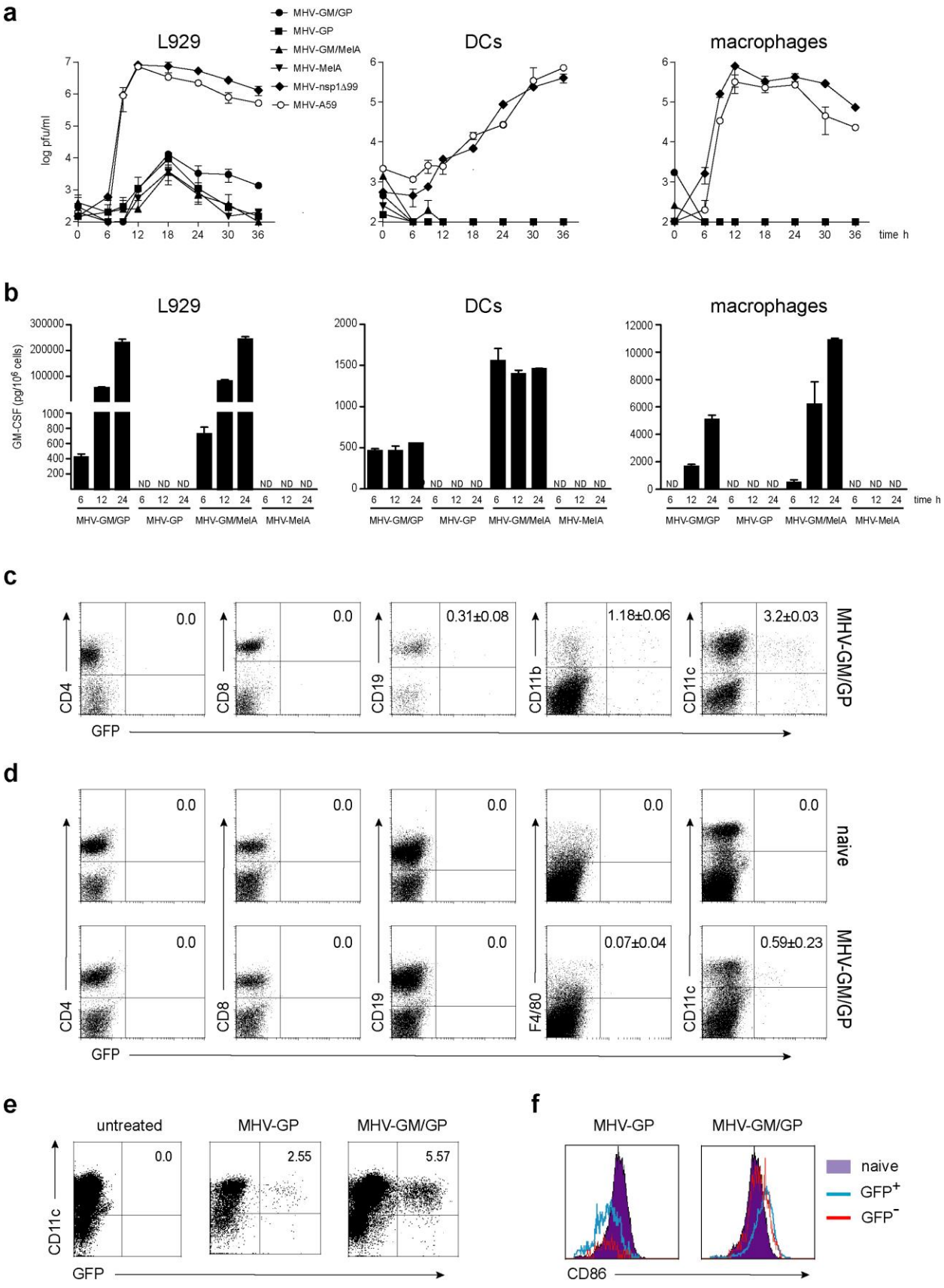

Figure 2. Antigen delivery to dendritic cells by MHV-based vaccine vectors. (A, B) 10^6 L292 cells, bone marrow derived DCs, or peritoneal macrophages were transduced or infected with the indicated MHV-based vectors, or

MHV-nsp1Δ99 and MHV-A59, respectively , and MHV-based vector titers and MHV titers (A) as well as production of GM-CSF (B) were determined. Values represent means ±SEM from triplicate cultures. ND, not detectable. (C-D) In vitro and in vivo transduction of splenocytes. (C) One million splenocytes or low density cell-enriched fractions (for cDC analysis) from B6 mice were transduced with MHV-GM/GP vector (moi=1). Cells were harvested 12 h later and stained for the indicated surface molecules (CD4 T cells, $CD4^+CD3^+$; CD8 T cells, $CD8^+CD3^+$; B cells, $B220^+CD19^+$; macrophages, $CD11b^+$; cDCs, $B220^-CD11c^+$) and subjected to FACS analysis. (D) B6 mice were infected i.p. with 10^6 pfu MHV-GM/GP or left untreated. Spleens were collected after 36 h, digested with collagenase and splenocytes or low density cells (for cDCs analysis) were stained for the indicated cell population as in (C) and subjected to FACS analysis. Dot plots are representative of 5 individual mice. Values in the upper right quadrant indicate mean percentages ±SD of GFP^+ cells for each population. (E-F) Stimulation of cDCs by GM-CSF expressing vectors. One million bone marrow-derived cDCs from B6 mice were infected with the indicated MHV-based vector (moi=1) or left untreated. Cells were harvested 12 h later, stained for CD11c and CD86 expression and subjected to FACS analysis. (E) Representative dot plots indicating the high transduction efficacy. Values in the upper right quadrant indicate of GFP^+ cells. (F) Expression of the DC activation marker CD86 on untreated $CD11c^+$ cells (grey shaded), on $CD11c^+GFP^+$ (thick black line), or $CD11c^+GFP^-$ (dotted line) in cells exposed to either MHV-GP or MHV-GM/GP.

To further determine the cellular tropism of MHV-based vectors, we transduced splenocytes from C57BL/6 (B6) mice with MHV-GM/GP in vitro and analyzed GFP expression by flow cytometry. As shown in figure 1c, neither $CD4^+$, nor $CD8^+$ T cells were susceptible to MHV-GM/GP transduction. In contrast, antigen presenting cells, such as B cells ($CD19^+$), macrophages ($CD11b^+$) and DCs ($CD11C^+$), displayed green fluorescence, indicative for MHV vector-mediated GFP expression. In order to evaluate if targeting of DCs and macrophages is also achievable in vivo, we injected (i.v.) 10^6 VLPs of MHV-GM/GP into B6 mice and analyzed GFP expression of splenocytes by flow cytometry. As shown in figure 2d, we could indeed detect GFP expression in $F4/80^+$ macrophages and $CD11c^+$ DC, but not in B or T cells. Finally, we assessed the effect of MHV vector-mediated GM-CSF expression on DC stimulation and activation. To this end, bone-marrow derived DCs were transduced (moi=1) with MHV-GP or MHV-GM/GP. Vector-mediated GM-CSF expression facilitated more efficient DC-transduction and permitted better survival of DCs, indicated by a higher recovery of living cells in the 12 h cultures (figure 2e). Furthermore, vector-mediated GM-CSF expression also resulted in DC activation and maturation, as shown by upregulation of CD86 on $CD11c^+$ cells (Fig. 2f). This effect was not only apparent in the MHV-GM/GP-transduced GFP^+ fraction, but also in the GFP^- fraction of non-transduced DCs, suggesting that GM-CSF expression by vector transduced DCs provides a substantial bystander effect on non-transduced DCs.

Antiviral CTL responses following coronavirus vector immunization

Infection with LCMV is characterized by a vigorous expansion of antiviral CTL and persistence of protective memory CTL (Wherry and Ahmed, 2004; Zinkernagel, 2002) which are directed against several epitopes. Two different epitopes are present in the gp_{33-41} region of the LCVM-GP that has been used in two of our constructs: the H2-D^b binding gp_{33-41} (Pircher et al., 1990) and the H2-K^b binding gp_{34-41} (Hudrisier et al., 1997).

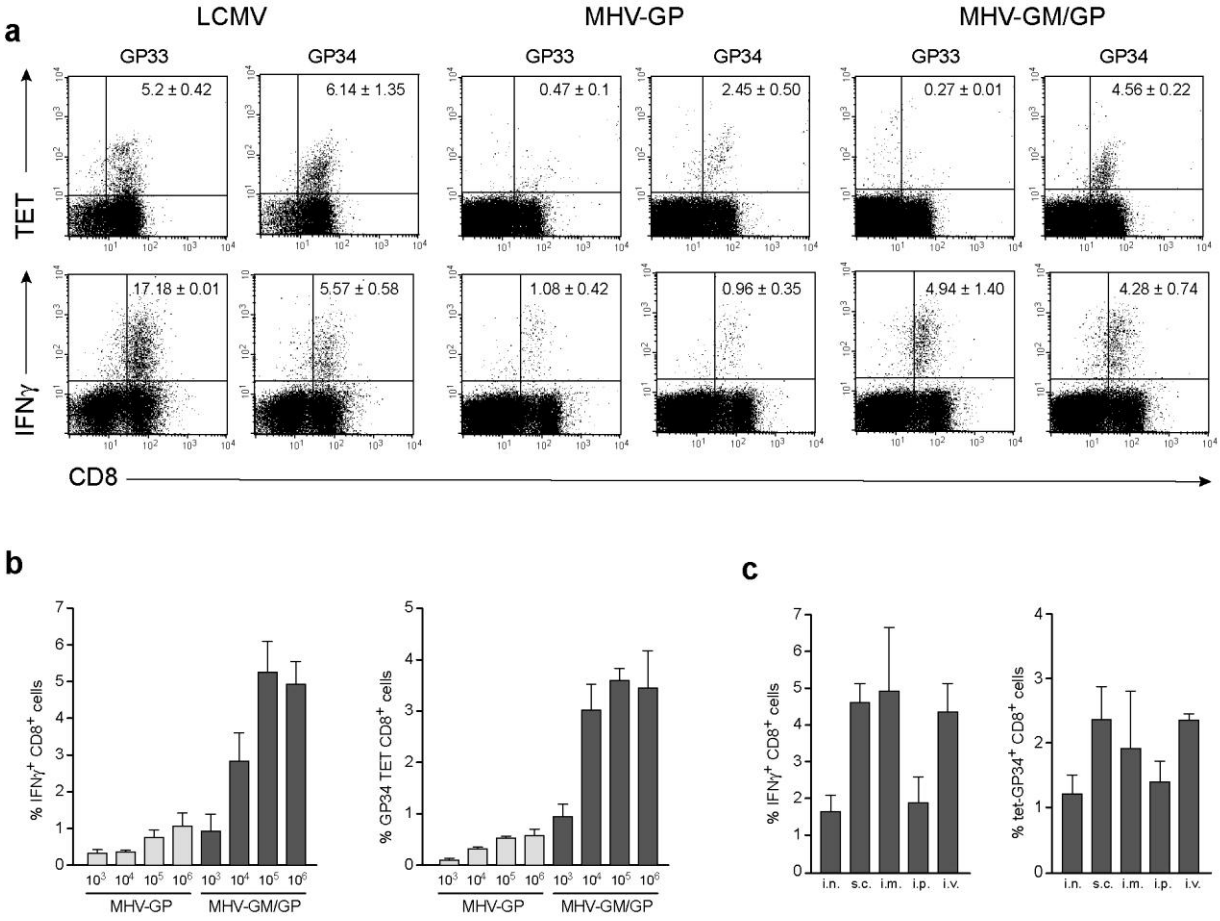

Figure 3. Evaluation of antiviral $CD8^+$ T cell responses. (A) B6 mice were infected i.v. with either 200 pfu LCMV as a control for vigorous CTL induction, 10^5 pfu MHV-GP, or 10^5 pfu MHV-GM/GP. At day 7 after infection splenocytes were analyzed for expression of CD8 and reactivity with H2-D^b/gp33-, and H2-K^b/gp34-tetramers. $CD8^+$ splenocytes were also analyzed for gp33- and gp34-specific IFN-γ production. Values in the upper right quadrants represent percentages of tet^+ cells ±SD (upper row) or percentages of IFN-γ^+ cells ± SD (lower row) in the CD8 T-cell compartment (n = 3 mice per group). (B) Efficacy of MHV-based vectors in inducing antiviral $CD8^+$ T cell responses. B6 mice were immunized i.v. with the indicated doses of MHV-GP or MHV-GM/GP and the percentage of H2-K^b/gp34-tetramer-binding $CD8^+$ T cells, and the percentage of gp34-specific IFN-γ producing $CD8^+$ T cells was determined on day 7 post immunization (n=6 mice per group, pooled from 2 different experiments). (C) Importance of the route on immunization. B6 mice were immunized with 10^5 pfu MHV-GM/GP

and the percentage of H2-K^b/gp34-tetramer-binding $CD8^+$ T cells, and the percentage of gp34-specific IFN-γ producing $CD8^+$ T cells was determined on day 7 post immunization (n=2-6 mice per group, pooled from 2 different experiments).

On day 7 post LCMV infection, significant numbers of $CD8^+$ T cells can be detected by MHC tetramer analysis and intracellular cytokine secretion (ICS) assay (Fig. 3a). It is important to note that the gp33 ICS records both gp33- and gp34-specific $CD8^+$ T cells. It appears that the processing of the GP-GFP transgene in the MHV-GP and MHV-GM/GP vectors permitted the preferential generation of gp34-specific $CD8^+$ T cells in a magnitude comparable to that seen during acute LCMV infection (Fig. 3a). The GM-CSF encoding MHV vector proved to be highly efficient in the induction of antiviral CTL, even at rather low doses of 10^4 pfu (Fig. 4b). Since the intermediate dose of 10^5 pfu of MHV-GM/GP led to optimal induction of gp34-specific CTL, we used this dose to assess whether application via different routes would influence the induction of transgene-specific CTL. As shown in Fig. 3c, all routes of immunization elicited robust $CD8^+$ T cell responses with s.c., i.m., and i.v. application being the most efficient means of application.

Protection against LCMV challenge requires high levels of appropriately activated $CD8^+$ T cells (Wherry et al., 2003; Zinkernagel, 2002). In order to assess the efficacy of MHV vector-based immunization for protection against viral challenge, B6 mice were immunized with graded doses of MHV-GP or MHV-GM/GP vectors and challenged 7 d later with LCMV. Comparable to the high efficiency of $CD8^+$ T cell induction (Fig. 3), mice were completely protected at a dose of 10^5 pfu (Fig. 4a). Remarkably, as few as 10^3 pfu of MHV-GM/GP led to a >3 log reduction of LCMV titers in spleens on day 4 post challenge (Fig. 4a). Protection against LCMV challenge was long-lasting because mice were still completely protected on day 65 post immunization with 10^5 pfu MHV-GM/GP (Fig. 4b). Moreover, the MHV-GM/GP vaccine elicited complete protection against i.p. challenge with LCMV-GP recombinant vaccinia virus (Fig. 4c). Likewise, immunization with MHV-GP provided a substantial protection against this heterologous viral infection (Fig. 4c). It is important to indicate that the MHV-based vaccine, even when GM-CSF was encoded by the vector, provided specific protection because replication of the unrelated VSV-G recombinant vaccinia virus (VV-INDG) was not affected (Fig. 4c).

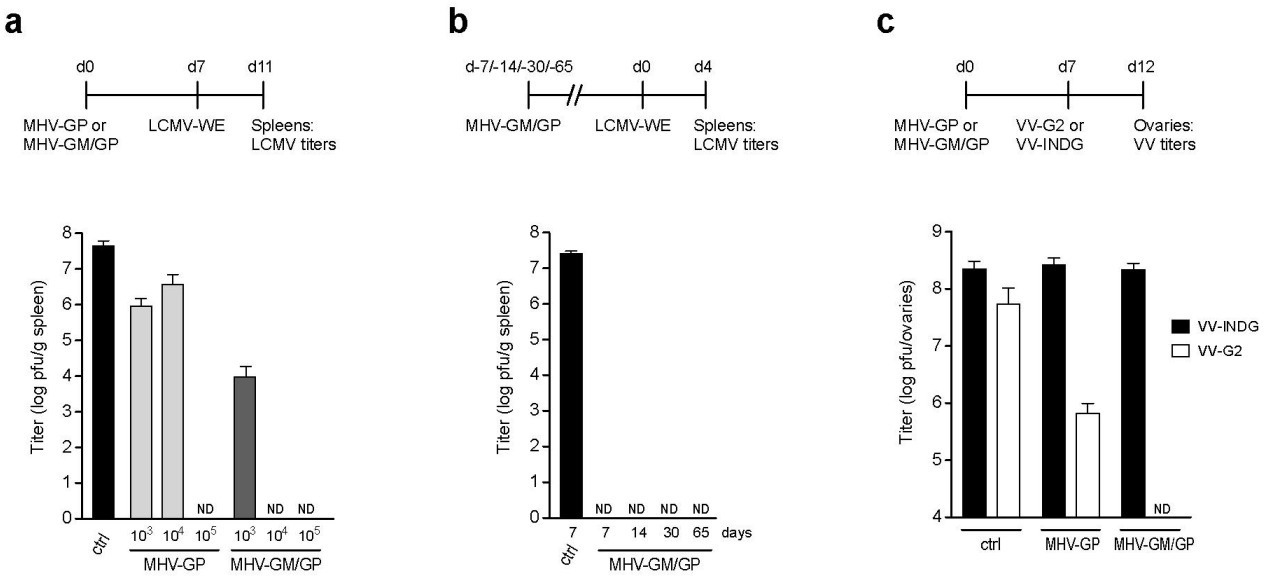

Figure 4. Induction of long-lasting protective immunity. (A) B6 mice were either left untreated (CTRL) or immunized (i.v.) with the indicated doses of MHV-GP or MHV-GM/GP. Seven days later, mice were challenged i.v. with 200 pfu LCMV-WE. Viral titers in spleens (means ±SEM) were determined 4 days after LCMV-WE challenge using focus-forming assay of MC57 cells (n = 4-6 mice per group, pooled from 2 different experiments). (B) Duration of protective antiviral immunity. B6 mice were immunized with 10^5 pfu MHV-GM/GP and challenged i.v. with 200 pfu LCMV-WE at the indicated time points Viral titers in spleens (means ±SEM) were determined 4 days after LCMV-WE challenge using focus-forming assay of MC57 cells (n = 4-6 mice per group). (C) Female B6 mice were either left untreated (CTRL) or immunized (i.v.) with the 10^5 pfu MHV-GP or 10^5 pfu MHV-GM/GP. Seven days later, mice were challenged i.p. with 2×10^6 pfu LCMV-GP recombinant (VV-G2), or vesicular stomatitis virus glycoprotein recombinant (VV-INDG) vaccinia viruses. Vaccina virus titers (means ±SEM) in ovaries were determined 5 days after challenge infection (n = 6 mice per group, pooled from 2 different experiments).

Taken together, these results revealed that the MHV-based vector system is highly efficient in generating protective antiviral immunity and that the incorporation of GM-CSF into the vaccine significantly augmented its immunogenicity, most likely via the activation of DCs (Fig. 2f).

Prophylactic and therapeutic antitumour immunization

In order to evaluate whether MHV vector-based vaccination elicits prophylactic and therapeutic tumour immunity, we resorted to a rapidly growing B16 melanoma model which provides compatibility with the LCMV-GP system through expression of a gp33 minigene (B16F10-GP) (Prevost-Blondel et al., 1998). I.v. injection of 5×10^5 B16F10-GP or parental B16F10 cells in control B6 mice resulted in metastatic growth of tumour cells in lungs (Fig. 5a).

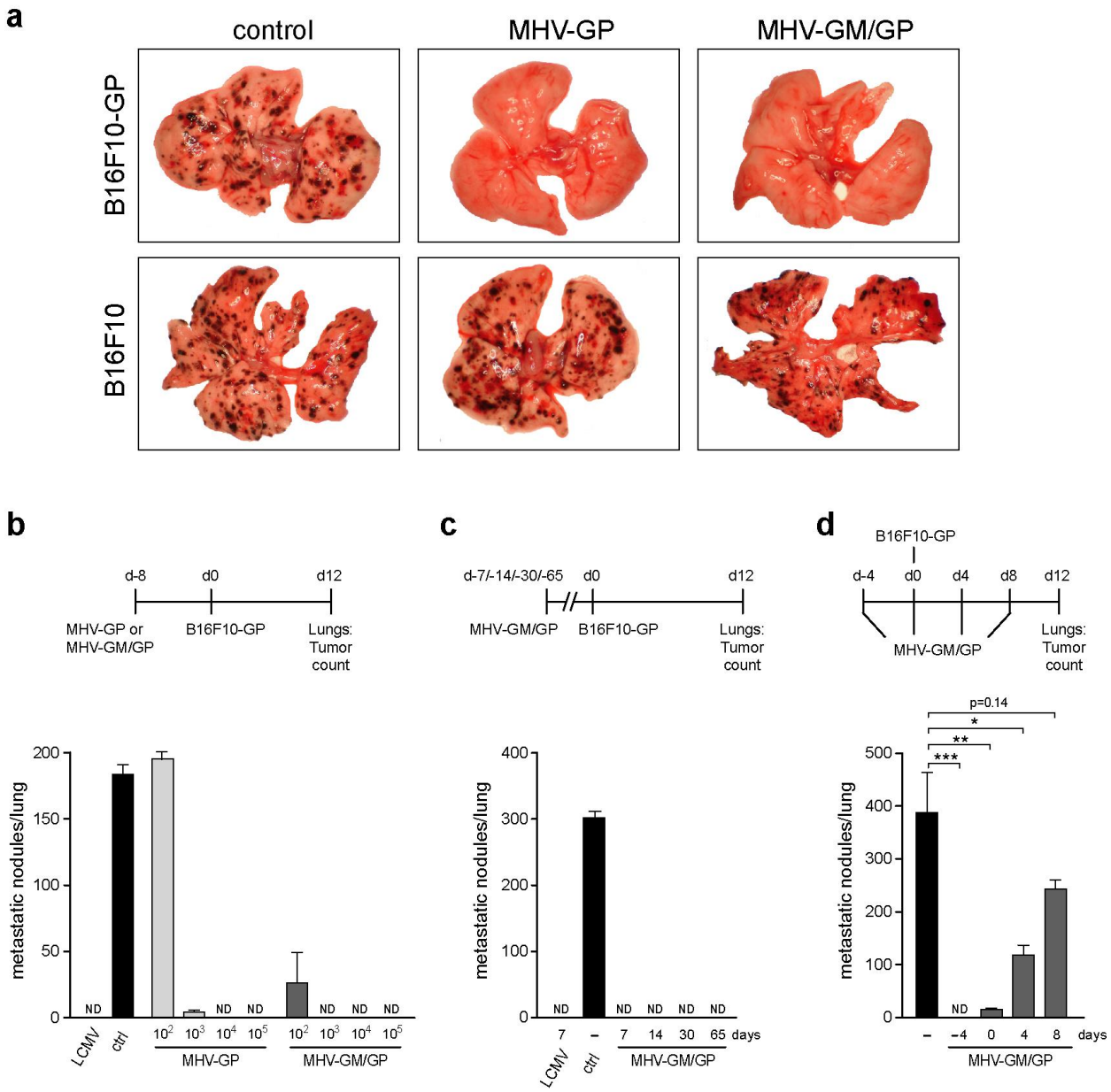

Figure 5. Prevention and immunotherapeutical treatment of metastatic melanoma. (A) B6 mice were either left untreated (CTRL) or immunized (i.v.) with either 10^5 pfu MHV-GP or MHV-GM/GP. Seven days later, mice were challenged with 5×10^5 LCMV gp33-recombinant B16F10-GP tumour cells or parental B16F10 tumours cells i.v. Tumor growth in lungs was recorded on day 12 post tumour challenge. Macroscopic pictures show representative lungs from 1 out of 3 mice per group. (B) Efficacy of MHV-based vectors in generating prophylactic tumour immunity. B6 mice were immunized i.v. with the indicated doses of MHV-GP or MHV-GM/GP and challenged 8 days later with 5×10^5 LCMV gp33-recombinant B16F10-GP tumour cells. Numbers of lung foci were determined on day 12. Data indicate means ±SEM (n=6 mice per group, pooled from 2 different experiments). (C) Duration of protective antitumour immunity. B6 mice were immunized with 10^5 pfu MHV-GM/GP and challenged at the indicated time points with 5×10^5 LCMV gp33-recombinant B16F10-GP tumour cells. Numbers of lung foci were determined on day 12 with data indicate means ±SEM (n=4-6 mice per group, pooled from 2 different experiments). (D) Therapeutic antitumour immunity. B6 mice received 5×10^5 LCMV gp33-recombinant B16F10-GP tumour cells

i.v. and were immunized with 10^5 pfu MHV-GM/GP i.v. either at the same day (day 0), or 4 or 8 days later. Numbers of lung foci were determined on day 12 post tumour inoculation; data indicate means ±SEM (n=4-6 mice per group, pooled from 2 different experiments). Statistical analysis was performed using Student's t test (***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; ns, $p>0.05$). ND, not detectable.

Immunization with either 10^5 pfu MHV-GP or 10^5 pfu MHV-GM/GP resulted in a complete block of B16F10-GP growth, whereas metastasis formation of the parental B16F10 cells was not affected (Fig. 5a). Application of graded doses of MHV-GP or MHV-GM/GP revealed the high efficacy of this vaccination approach in the prophylactic setting, i.e. 10^4 MHV-GP or only 10^3 pfu MHV-GM/GP were sufficient to completely block growth of the melanoma cells (Fig. 5b). A long-lasting memory response had been generated following MHV-GM/GP immunization that provided full protection against B16F10-GP challenge (Fig. 5c). Moreover, the potent $CD8^+$ T cell response elicited through MHV-GM/GP immunization mediated therapeutic tumour immunity (Fig. 5d), i.e. the tumour burden in lungs of B6 mice was significantly reduced even when the vaccine was applied after the tumours had started to form metastatic nodules, indicating that $CD8^+$ T cell responses elicited by the novel MHV vectors can exert forceful antitumour activity.

To further substantiate the finding that MHV vectors can induce potent and relevant antitumour $CD8^+$ T cell responses, we employed the human HLA-A2-restricted Melan-A/MART1 system where immune responses against the Mel-A_{26-35}A27L analog peptide (Valmori et al., 1999) can be followed in HLA-A2.1 transgenic (A2DR1) mice (Pajot et al., 2004). A2DR1 mice were immunized i.v. with either 10^5 pfu MHV-MelA or 10^5 pfu MHV-GM/MelA, and Mel-A-specific $CD8^+$ T cell responses were recorded using tetramer analysis and ICS. As shown in Fig. 6a, both vectors elicited substantial $CD8^+$ T cell responses. Time course experiments following i.v. application of MHV-GM/MelA revealed a strong global expansion of $CD8^+$ T cells (nearly 10-fold) and a massive expansion of Mel-A-specific $CD8^+$ T cells with 6×10^6 cells per spleen being tetramer-positive and 2×10^6 cells per spleen secreting IFN-γ following short-term in vitro restimulation (Fig. 6b). The Mel-A-specific $CD8^+$ T cell population showed a typical contraction after day 7 and a stable memory population on day 28 post immunization (Fig. 6b). During the acute phase following MHV-GM/GP immunization, >90% of the Mel-A-specific $CD8^+$ T cells had down-regulated CD62L (Fig. 6c). As expected, memory $CD8^+$ T cell re-acquired CD62L-expression indicating establishment of a central memory $CD8^+$ T cell population. Overall, these results underline that the coronavirus-based vaccination

approach, particularly in combination with the immunostimulatory cytokine GM-CSF, provides efficient means for the induction of protective $CD8^+$ T cell responses.

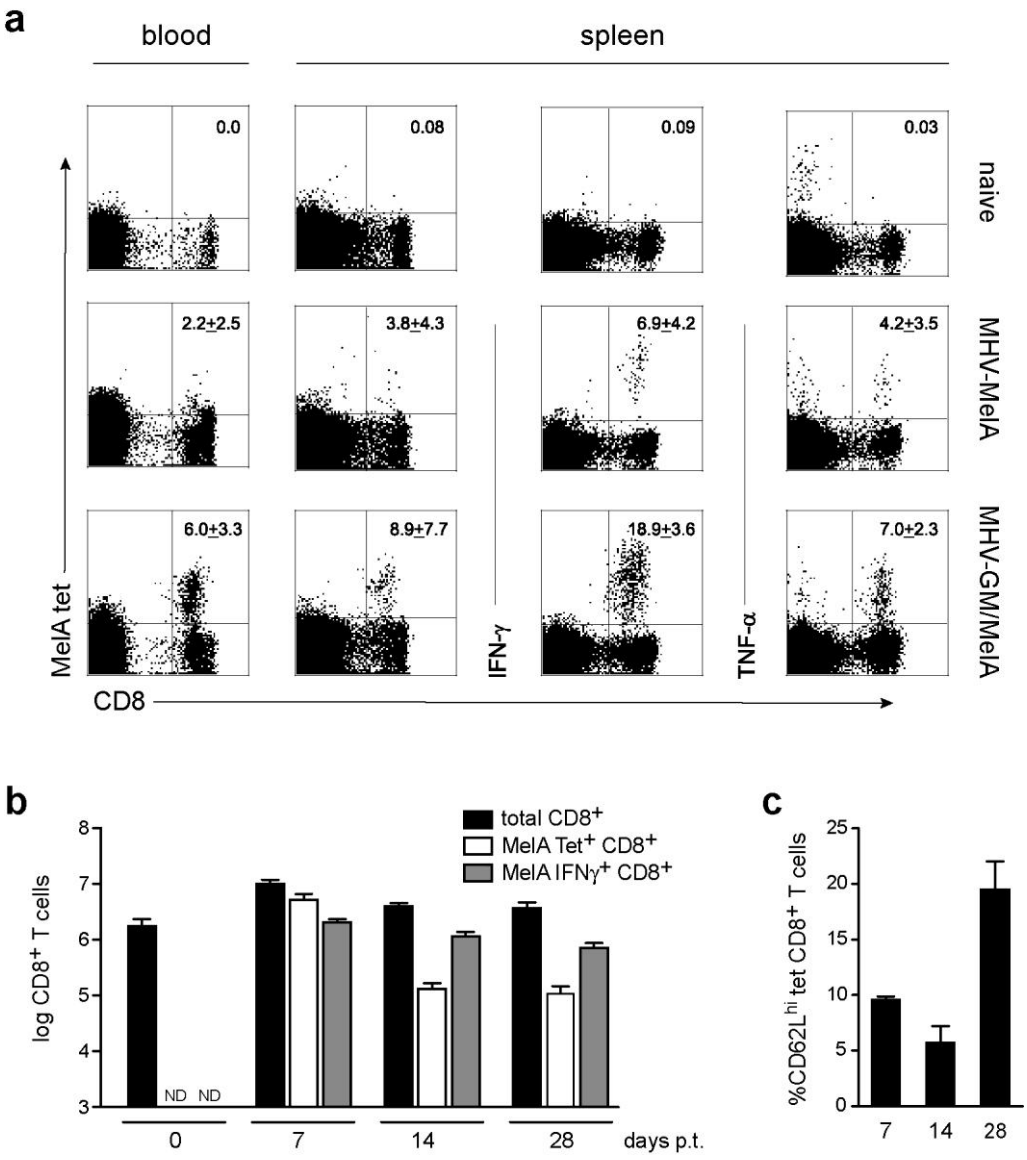

Figure 6. Assessment of anti-Melan-A/MART1 $CD8^+$ T cells in A2DR1 mice. (A) Transgenic mice expressing the human HLA-A2.1 molecule were immunized i.v. with 10^5 pfu MHV-MelA or MHV-GM/MelA. At day 7 post infection, splenocytes and mononuclear blood cells were analyzed for expression of CD8 and reactivity with HLA-A2/Mel-$A_{26\text{-}35}$A27L tetramers, and for Mel-$A_{26\text{-}35}$A27L-specific IFN-γ production. Values in the upper right quandrants represent mean percentages of tet^+ cells ±SD in blood and spleen, or percentages of IFN-γ^+ cells ± SD in the CD8+ T-cell compartment (*n* = 3 mice per group). (B) Time course of Mel-$A_{26\text{-}35}$A27L-specific $CD8^+$ T-cell responses in A2DR1 mice following i.v. immunization with 10^5 pfu MHV-GM/MelA. Total numbers of $CD8^+$ T-cells, tetramer-binding Mel-$A_{26\text{-}35}$A27L-specific $CD8^+$ T-cells, and Mel-$A_{26\text{-}35}$A27L-specific IFN-γ^+CD8^+ T-cells cells were determined at the indicated time points post immunization (means ±SD, n = 3 mice per group). (C) Differentiation of tetramer-binding Mel-$A_{26\text{-}35}$A27L-specific $CD8^+$ T-cells as determined by CD62L expression at

the indicated time points post immunization (means ±SD, n = 3 mice per group). Data in A-C are from one representative experiment out of three.

Discussion

This study describes a novel vaccination approach that facilitates delivery of viral or tumour antigens to DCs in vivo. Concomitant immunostimulation – here via the cytokine GM-CSF – was achieved through targeted delivery by the same viral vector. Single immunization with only 10^4 – 10^5 particles was sufficient to elicit (i) vigorous expansion and optimal differentiation of $CD8^+$ T cells, (ii) protective and long-lasting antiviral immunity, (iii) prophylactic and therapeutic tumour immunity.

Targeting of antigen to DCs in vivo can be achieved by several means (Tacken et al., 2007) whereby the use of viral vectors appears to be the superior strategy to elicit innate activation of the immune system and optimal induction $CD8^+$ T cells. However, many virus vector systems are still limited in their ability to induce broad and long-lasting immune responses. For example, recombinant adenoviruses have been studied intensively as vaccine candidates mainly because they can be produced to high titers. Nevertheless, high doses of recombinant adenovirus vectors have to be applied to induce an immune response, most probably because they target antigens mainly to non-lymphoid organs such as the liver (Engelhardt et al., 1994; Yang et al., 2006). In contrast to viral vectors based on DNA viruses (Trono, 2003), positive-stranded RNA virus-based vectors that replicate in the cytoplasm are considered as safe vectors. The safety is well documented for vectors based on widely used vaccine strains such as poliovirus (Crotty et al., 1999) or virus like particles (VLPs) that contain replicon RNAs devoid of structural genes (Davis et al., 2000; Harvey et al., 2003). Although some of these vectors are able to target DC, their cloning capacity is generally restricted and the expression of multiple tumour antigens and/or immunostimulatory cytokines is limited.

A number of reasons probably account for the high potency of the novel coronavirus-based vaccine. Following the reasoning of several recent publications (Hickman et al., 2008; Junt et al., 2007; Junt et al., 2008), it is the pronounced tropism of the MHV-based vectors for transduction of not only DCs, but also macrophages within secondary lymphoid organs that guarantees efficient activation of primary $CD8^+$ T cell responses. In vivo imaging studies have shown that $CD169^+CD11c^+$ DCs at the subcapsular sinus of lymph nodes efficiently present viral antigen to naïve T cells (Hickman et al., 2008). Likewise, $CD169^+$ macrophages at the same location are able to collect viral antigen from the lymph and present antigen to follicular B cells (Junt et al., 2007). Morevover, a recent study from our laboratory has revealed that type IFN-mediated protection of DCs and macrophages from cytopathic effects of MHV infection is essential to buy time for mounting a protective $CD8^+$ T cell response (Cervantes-Barragan, in

press). It remains to be resolved which factors – besides the presence of the MHV receptor on DCs (Zhou and Perlman, 2006) – confers the preferential infection of the relevant antigen presenting cells within secondary lymphoid organs. For the further adaptation of the coronavirus-based vectors to the human system, some of the essential parameters have been clarified, i.e. the receptor of HCoV 229E is expressed mainly on monocytes and DCs within secondary lymphoid organs (Summers et al., 2001) and recombinant HCoV 229E transduces human DCs irrespective of their maturation status (Thiel et al., 2003).

A second major advantage of the coronavirus-based vaccination strategy is the large cloning capacity that offers the possibility to incorporate immunostimulatory cytokines. GM-CSF encoding MHV-based vectors led to strong production of this cytokine in both macrophages and DCs in vitro. In vivo, however, GM-CSF expression appeared to be restricted to locally transduced cells since elevated GM-CSF levels could be observed neither in sera, nor in spleen homogenates of i.v. immunized animals (data not shown). It is most likely that GM-CSF-induced changes within the microenvironment of transduced macrophages and DCs are decisive for the optimal induction of maximal effector and memory $CD8^+$ T cell responses. Indeed, it is the optimally stimulated expression of co-stimulatory molecules on antigen presenting cells such as DCs and macrophages together with sufficient innate immune stimulation, that determines the primary expansion and the maintenance of antiviral $CD8^+$ T cells (Harty and Badovinac, 2008). Accordingly, such non-TCR signals ("signal 2 and 3") are considered as key components of rationally designed vaccines (Appay et al., 2008). The lack of such optimally composed stimuli in a vector-based vaccine most likely requires substantially increased doses to achieve efficacy, as for example in vaccination with latest versions of DC-adapted lentivirus vectors: application or $5–10\times10^7$ particles was required to achieve significant expansion of ovalbumin-specific $CD8^+$ T cells (Yang et al., 2008).

Taken together, the efficient generation of protective immunity by the coronavirus-based vaccine, shown as long-lasting memory against viral challenge and induction of both prophylactic and therapeutic tumour immunity, indicates that this inherently safe RNA viral vector-strategy harbors a great potential for future development.

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7 Discussion and future direction

The modification of attenuated viruses and bacteria to carry genes encoding antigenic-proteins from other pathogenic organisms in their genome is greatly facilitated by the identification and availability of immunogenic antigens. Vectors provide an efficient means to deliver antigens from pathogens which themselves might be considered as high risk despite attenuation and hence may never be allowed for vaccination. Viral vectors provide several advantages over naked nucleic acid and immunogenic peptides in their heightened strategy to deliver antigens and or cytokines to specific target cells. For some reasons however, many viral vectors are still limited in their ability to induce a broad and long-lasting immune response. Furthermore the safety concerns of DNA based vectors such as adeno-associated, retroviral or lentiviral vectors raise a number of questions with regards to integration and possible modification of the host cell genome (Dobbelstein, 2003; Trono, 2003). Though recombinant adenoviruses have been extensively studied because they easily yield high titers, the extremely high doses required for immunization in order to induce an immune response indicate that they may be targeting their genetic load to the wrong organs such as the liver (Engelhardt et al., 1994; Yang et al., 2006). In principle the use of positive stranded-RNA vectors with a replication that is completely restricted to the cytosol of the infected cells is considered very safe because it is unlikely that viral derived sequences integrate into the host cell genome. Additionally, this safety profile is properly substantiated for vectors based on widely used vaccine strains like poliovirus (Crotty et al., 1999) or VLPs that are devoid of all structural genes and contain only replicon RNAs. Furthermore several such vectors can target their genetic load to DCs but their limited cloning capacity may be a handicap restricting the cloning and simultaneous expression of multiple antigens. Coronaviruses possess a number of intrinsic properties making them to pose as very promising vaccine vector candidates.

7.1 What has been done

We have developed a new class of multigene-RNA vaccine vectors based on mouse hepatitis coronavirus. The successfull rescue of these vectors necessitates a special packaging cell line that is capable of providing the necessary structural protein(s) needed for efficient propagation in trans. We could demonstrate efficient targeting of pAPCs such as dendritic cells and macrophages. Vector-mediated heterologous protein expression in the target cells was evaluated using standard protocols. This efficient targeting and antigen expression provides the proof of principle that it is possible to insert the antigen of interest in MHV-based vector genome

and efficiently target the genetic cargo to professional determinants of the immune response. Furthermore, these MHV-based VLPs have demonstrated a very strong and specific induction of CTL-responses towards the encoded GP33 CTL epitope as well as towards the human melan-A CTL epitope.

The specific CTL response induced by MHV-based VLPs was very high after a single immunization with only 10^5 VLPs as compared to other vector systems like the modified vaccinia Ankara (MVA) wherein doses of 10^7-10^8 particles are usually utilized to achieve a much lower response. In fact vaccine strategies such as those based on attenuated vaccinia recombinant NYVAC (Tartaglia et al., 1992) and the modified vaccinia Ankara have entered into several clinical trials. Foreign genes such as those for HIV env or gag-pol are encoded by these vectors (Robinson et al., 2006); however, clinical trials have indicated that several vectors and adjuvants may be needed to achieve efficacious responses in humans towards the encoded antigens (Cebere et al., 2006; Mwau et al., 2004; Robinson et al., 2006). Additionally, lentivirus based vector particle expressing the same human melan-A CTL epitope as encoded in MHV-based VLPs, needed 4 x10^6 particles to achieve a very low CTL response as against the single dose of 10^5 MHV-based VLPs.

The stringency for vaccine safety has resulted in several viral vaccine vectors with only very low immunogenicity necessitating in most cases two to three boosts in order to increase potency (Cebere et al., 2006). Therefore, these MHV-based vectors reported here have been designed to demonstrate a high biosafety profile when compared to attenuated viral vaccines. Furthermore, we did not employ any boosting regimen simply because the immune response observed was strong enough to provide complete protection against a pathogenic high dose virus challenge and against a lethal rapidly metastasizing tumour model.

Several optimization strategies exist to increase the immunogenicity of a viral vaccine vector in addition to altering the vector itself. For instance immunogenicity can be improved following a careful systematic evaluation of critical steps such as (i) the route and method of vaccine delivery (ii) the number of vaccinations and their timing (iii) the dose utilized and lastly (iv) the choice of adjuvant which may make a difference. In this study, we have examined the outcome of different routes of immunization using MHV-based VLPs and the observed result demonstrated that we could virtually immunize by any of the chosen routes and the induction of immune response will be equally protective. Amazing, is the fact that, our system induces one of the highest immune induction using the intramuscular or intravenous routes. It must be stressed here that in human immunizations strategies the intramuscular as well as intravenous routes are the most preferred vaccination routes in standard protocols.

In fact, the expression of the cytokine GM-CSF in our constructs indicated enhanced T cell response as compared to vectors that do not encode this cytokine. This means the manipulation of the immune outcome is possible especially in a situation where we are able to effectively target the vaccine to specific immune activators such as DCs and macrophages. Additionally, we will exploit this system by using different cytokines either on their own or in combination to induce maturation and influence the stimulatory capacity of specific immune components.

The maturation and proper stimulation of T cells is crucial for the generation of potent T cell responses (Steinman et al., 2003) meanwhile the ability of DCs to drive antigen-specific immunity can be modulated by adjuvants. Furthermore, most adjuvants work through the activation of innate immune activators such as NK cells and DCs. For instance, Toll-like receptor (TLRs) ligands such as bacteria DNA are capable of stimulating DCs (Sparwasser et al., 1998) leading to a more improved activation of tumour specific CTL response (Ludewig et al., 2000). A major setback of TLR ligands as immunological adjuvants is their broad and systemic activity that may cause unwanted side effects (Heikenwalder et al., 2004). Cytokines on the other hand are molecules having well defined effects on both the innate and adaptive immune cells. In fact GM-CSF is an important mediator of DC maturation (Heufler et al., 1988) and survival (Witmer-Pack et al., 1987). Furthermore the fms-liks tyrosine kinase 3-ligand (Flt3-L) is a cytokine with strong stimulatory effect leading to DC proliferation and accumulation in vivo (Maraskovsky et al., 1996). Interestingly both GM-CSF (Chiodoni et al., 1999; Nakamura et al., 2002) and Flt3-L (Lynch et al., 1997) have been used successfully as adjuvants in antitumour vaccine. Other cytokines function more downstream of the immune activation cascade. The T cell stimulatory cytokines IL-2 and IL-15 for instance, are known to enhance proliferation of T cells as well as facilitate the differentiation of CTL. However, there are distinct points of action where these cytokines impact on T cell differentiation despite sharing a common gamma chain and the IL-2/IL-15 receptor. IL-2 is highly important during the early expansion of T cells and through activation induced cell death it leads to the limitation of the T cell overshoot (Marks-Konczalik et al., 2000). IL-15 on its part is important for the survival of high-affinity T cells during the memory phase (Ku et al., 2000) and thereby ensures the maintenance of protective T cell memory response. Therefore, it is very important to note that both IL-2 and IL-15 are highly efficient adjuvants that greatly enhance tumour-specific T cell responses (Waldmann, 2006). The generation of highly efficient T cell responses is possible if these cytokines are incorporated either singly or in combination as adjuvants in an antigen-specific tumour vaccine. Using the MHV-based vector system reported here for example, we will clone various cytokines into the

vector genome and we will systematically study their specific effects and in addition assess any synergistic effects on immunologically important target cells.

7.2 Possible way out of vector-mediated neutralizing antibody or pre-existing immunity

Significant efforts are being expended to demonstrate whether the pre-existing immunity weakens the effectiveness of vectors such as with adenovirus-based vectors. For instance, the adenovirus serotype 5 is the most explored in terms of vaccine vectors but the seroprevalence in the world is high against this particular type of the virus (Shiver and Emini, 2004). Since our vaccine strategy is based on a mouse pathogen, we strongly believe that there is no pre-existing immunity in the human population towards this virus. Tropism and species specificity is dictated by the coronavirus spike glycoprotein therefore, the humanization of the MHV-based VLPs to target human cells will necessitate the altering of the MHV spike protein by chimerisation. This will be achieved by exchanging the ectodomain of the MHV spike protein with that of a potential HCoV with the lowest prevalence in the human population. Employing the strategy of swapping the ectodomain of the MHV-based VLPs with that of a HCoV, we can additionally, avoid vector-mediated neutralizing antibodies in case a boost is absolutely needed. Furthermore, since coronaviruses are responsible for most of the human common cold, a mucosal administration of humanized particles could be considered as a strategy to overcome systemic antivector preimmunity. In fact mucosal administration of measles and measles-rubella vaccines has been demonstrated to be safe and more efficient than subcutaneous vaccination in preimmunized humans (Dilraj et al., 2000; Gans et al., 2003).

7.3 Relevance of this work to science

This study has demonstrated that the generation of coronavirus-based-VLPs is feasible and that the transgenes expressed by these replication-competent but propagation deficient vectors can be targeted to DCs and the resulting immune response is protective. Furthermore, MHV being the most studied coronavirus in the laboratory coupled to the availability of a variety of properly characterized small animal models (the mice) and the demonstration that MHV-based VLPs can infect murine DCs, indicate that this model will serve as a valuable tool for the development, evaluation and characterization of coronavirus-based vaccine vectors geared towards human disease.

8 Apendix

8.1 References

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8.2 Abbreviations

AAV	Adeno-associated virus
ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired immunodeficiency syndrom
ALT	Alanine aminotransferase
APC	Antigen presenting cell
BCV	Bovine coronavirus
Bp	Base pairs
C-terminal	Carboxy terminal
CAT	Chloramphenicol acetyltransferace
Ceacam1a	Carcino-embryonic antigen related cell adhession molecule 1°
CTL	Cytolytic T lymphocytes
CCR7	Chemokine receptor 7
CDC	Complement-dependent cytotoxicity
cDNA	Complementary deoxyribonucleic acid
CMV	Cytomegalovirus
CNS	Central nervous system
DC	Dendritic cell
DNA	Deoxyribonucleic acid
E	Envelope glycoprotein
EBV	Epstein-Bar virus
ELC	EBV-induced molecule 1 ligand chemokine
FPV	Fowl-pox virus
FIPV	Feline infectious peritonitis virus
Fc	Fragment crystallizable
FMDV	Foot and mouth disease virus
GP	Glycoprotein
GM-CSF	Granulocyte macrophage colony stimulating factor
GFP	Green fluorescence protein

GTP	Guanine phosphoribosyltransferase
H'APN	Human aminopeptidase N
HA1	Hemagglutinin fusion protein 1
HE	Hemagglutinin-estarase
HCV	Human coronavirus
HCoV	Human coronavirus
HLA	Human leukocyte antigen
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
IBV	Infectious bronchitis virus
JHM	John Howard Müller
LCMV	Lymphocytic choriomeningitis virus
LUC	Luciferase
LAMP	Lysosomal associated membrane protein-3
M	Membrane glycoprotein
MHV	Mouse hepatitis virus
MHC	Major histocompatibility complex
MMR	Measles mumps rubella
MIP3	Macrophage inflammatory protein
MV	Measles virus
mRNA	Messenger ribonucleic acid
N	Nucleocapsid
NKT	Natural killer T cell
Nsp1	Non-structural protein 1
ORF	Open reading frame
pAPC	Professional antigen presenting cell
P.i	Post infection
PFU	Plaque forming unit

pDCs	Plasmacytoid dendritic cells
PRL	Pattern recognition ligand
RNA	Ribonucleic acid
S	Spike
SIV	Simian immunodeficiency virus
SIN	Sindbis virus
SFV	Semliki forest virus
SLC	Secondary lymphoid tissue chemokine
SARS	Seviere acute respiratory syndrome
TCR	T cell receptor
T_H	T helper
TNF-β	Tumour necrosis factor-beta
TCV	Turkey coronavirus
TGEV	Transmissible gastroenteritis virus
TLRs	Toll like receptors
Treg	T regulatory
TRS	Transcription regulatory sequence
T_{EM}	T effector memory
T_{CM}	T central memory
VLPs	Virus like particles
VSV	Vesicular stomatitis virus
VSV-G	Vesicular stomatitis virus glycoprotein
VEEV	Venezuelan equine encephalitis virus

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